#2

PI 1065105

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office

September 12, 2003

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE.

APPLICATION NUMBER: *60/400,900*
FILING DATE: *August 02, 2002*
RELATED PCT APPLICATION NUMBER: *PCT/US03/24322*

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

M. Tarver

M. TARVER
Certifying Officer

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

08-05-02

Substitute Form PTO/SB/16 (11-95)
Approved for use through 01/31/98, OMB 0651-0037
Patent and Trademark Office; US DEPARTMENT OF COMMERCE

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 C.F.R. § 1.53(c).

Docket Number	00786/432001	Type a plus sign (+) inside this box --->	+

INVENTOR(S)/APPLICANT(S)			
LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY)
Seed	Brian		Boston, Massachusetts
Yang	Yi		Cambridge, Massachusetts

TITLE OF THE INVENTION (280 characters max)

METHODS FOR THE PRODUCTION OF CELLS AND MAMMALS WITH DESIRED GENETIC MODIFICATIONS

CORRESPONDENCE ADDRESS

Karen L. Elbing, Ph.D.
Clark & Elbing LLP
101 Federal Street
Boston, MA 02110

Customer No.: 21559

STATE	MA	ZIP CODE	02110-2214	COUNTRY	USA

ENCLOSED APPLICATION PARTS (check all that apply)

☒ Specification	Number of pages: 42	☒ Petition to Accept Color Drawings	Pages: 2
☒ Claims	Number of pages: 7	☒ Petition Fee	Check Amount: $130
☒ Abstract	Number of pages: 1	☒ Return Postcard	1
☒ Black & White Drawings	Number of sheets: 16		
☒ Color Drawings	Number of sheets: 15		

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☒ A check or money order is enclosed to cover the filing fees. ☒ The Commissioner is hereby authorized to charge filing fees not covered and apply any credits to Deposit Account Number: 03-2095	FILING FEE AMOUNT	$80.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☐ No.

☒ Yes, the name of the U.S. Government agency and the Government contract number are: NIH AI27849, AI46731, and HL66678

21559
PATENT TRADEMARK OFFICE

☒ Applicant claims small entity status under 37 C.F.R. § 1.27.

Respectfully submitted,

SIGNATURE: Vicki L. Healy DATE: August 2, 2002

TYPED OR PRINTED NAME: Karen L. Elbing, Ph.D. Vicki L. Healy REGISTRATION NO.: 35,238

Reg. No.: 48,343

60400900 .000202

Certificate of Mailing

Date of Deposit. August 2, 2002

Label Number: EL 947117528 US

I hereby certify under 37 C.F.R. § 1 10 that this correspondence is being deposited with the United States Postal Service as "Express Mail Post Office to Addressee" with sufficient postage on the date indicated above and is addressed to BOX PROVISIONAL APPLICATION, Assistant Commissioner for Patents, Washington, D.C. 20231.

Guy E. Beardsley
Printed name of person mailing correspondence

Signature of person mailing correspondence

PROVISIONAL APPLICATION

UNDER 37 C.F.R. § 1.53(c)

APPLICANTS: BRIAN SEED
YI YANG

TITLE : METHODS FOR THE PRODUCTION OF CELLS AND MAMMALS WITH DESIRED GENETIC MODIFICATIONS

METHODS FOR THE PRODUCTION OF CELLS AND MAMMALS WITH DESIRED GENETIC MODIFICATIONS

Background

In general, the invention features novel methods for rapidly generating cell lines and mammals with site-specific genetic modifications of interest.

As the human genome sequencing effort approaches completion, much of the research focus has shifted from physical mapping of the genome to functional annotation of its contents. The challenge lies in evolving comprehensive approaches to efficiently define gene functions *in vivo*. The murine system is ideal for functional genomics because of the underlying biological similarity between human and mouse, the rapid advances in mouse genome sequencing, and the ability to genetically manipulate mouse embryonic stem (ES) cells.

Although gene disruption in mice has been widely used for functional analyses of genes *in vivo*, traditional procedures for generating site-specific gene knockout mice are generally time-consuming and labor-intensive. For these traditional methods, the targeting construct usually contains 6-10 kb of genomic sequence from the gene of interest with, e.g., a *neomycin-resistant* (*neo*^{r}) gene inserted into the coding region and a herpes-virus *thymidine kinase* (*tk*) gene placed at one end (Fig. 1A). The targeting construct is electroporated into ES cells and replaces the endogenous locus by homologous recombination. After verification by genomic DNA Blotting, ES cells bearing the mutant locus are injected into a mouse blastocyst to generate chimera mice. Finally, heterozygous and eventually homozygous mutant mice are obtained from the breeding of chimeric animals.

To make the targeting construct often requires fine restriction enzyme mapping of the gene and multi-step cloning, which is a long and tedious process. In order to prevent expression of a partial gene, which can result from alternative splicing, the insertion site should be as close to the translation initiation ATG as possible. This is often hindered by the lack of a convenient cloning site around the desired region. Furthermore, the length of homologous fragments that can be included in the targeting construct is often limited, resulting in low homologous recombination frequency.

Thus, improved methods are needed to more efficiently generate genetically modified cell lines and non-human mammals.

Summary of the Invention

The purpose of the present invention is to provide improved methods for genetically altering cells. In particular, these methods involve generating a modified artificial chromosome by homologous recombination between an artificial chromosome containing a nucleic acid of interest (e.g., a bacterial or yeast artificial chromosome containing a genomic insert from the same genus or species as the cell to be subsequently modified) and a nucleic acid (e.g., a linear DNA molecule) containing a region or the entire nucleic acid of interest with a desired mutation. In some embodiments, the artificial chromosome is a bacterial artificial chromosome (BAC) or a P1-based artificial chromosome (PAC), and the homologous recombination occurs in bacteria, or the artificial chromosome is a yeast artificial chromosome (YAC), and the homologous recombination occurs in yeast. The resulting modified artificial chromosome with the desired mutation is introduced into one or more cells (e.g., mammalian cells such as mouse, ungulate, or human cells), and the genetically modified cells in which homologous recombination occurs between the artificial chromosome and corresponding region of an endogenous chromosome in the cell are selected.

In some embodiments, the cell is modified to express a reporter gene under the control of an endogenous promoter of interest. In other embodiments, the cell is modified such that an exogenous nucleic acid encoding a detectable protein is operably linked to an endogenous nucleic acid encoding a protein of interest, thereby generating a genetically modified cell that expresses a fusion protein having the detectable protein and the protein of interest or a fragment thereof. These modified cells can be used in a variety of screening assays to identify candidate compounds that modulate the activity of the promoter of interest or the expression of the protein of interest. Additionally, these cells can be used in any standard method for the generation of mammals with one or more desired genetic modifications. These mammals are also useful in screening assays to identify compounds for the treatment or prevention of disease.

Accordingly, in a first aspect the invention provides a method of producing a genetically modified cell. This method involves inserting into one or more cells an artificial chromosome (e.g., a linear BAC, PAC, or YAC) having a cassette which includes a first region of homology having substantial sequence identity to a first region of an endogenous chromosome of the cell(s), a selectable marker, and a second region of homology having substantial sequence identity to a second region of the endogenous chromosome. Homologous recombination occurs between the artificial chromosome and the endogenous chromosome (e.g., homologous recombination between the first region of homology and the first region of the endogenous chromosome and homologous recombination between the second region of homology and the second region of the endogenous chromosome), and the cassette is integrated into the endogenous chromosome of one or more cells. One or more cells are selected in which the homologous recombination occurs, thereby selecting one or more genetically modified cells.

In preferred embodiments, the artificial chromosome having the cassette is produced by a culturing a cell (e.g. a bacterial or yeast cell) that has (i) a linear DNA molecule having the cassette and (ii) an artificial chromosome having

nucleic acid that is substantial identical to the first and second regions of homology (e.g., a BAC, PAC, or YAC having a genomic insert from the same genus or species as the cells to be subsequently modified) under conditions that result in homologous recombination between the linear DNA molecule and the artificial chromosome. In particular embodiments for generating the artificial chromosome with the cassette, the linear DNA molecule is introduced into the cell by transformation. In other embodiments, the linear DNA molecule is introduced into the cell by insertion of a circular vector having the sequence of the linear DNA molecule into the cell and cleavage of the vector to generate the linear DNA molecule inside the cell. It is also contemplated that the homologous recombination between the artificial chromosome and the linear DNA molecule can occur in an *in vitro* sample that has a recombinase that catalyzes homologous recombination. In preferred embodiments, the first and second regions of homology in the linear DNA molecule are less than 2,000, 1,000, 500, 250, 200, 100, 75, 50, or 25 nucleotides in length. In other embodiments, the first and second regions of homology are between 2,000 and 10,000 nucleotides in length, such as between 2,000 and 5,000 nucleotides or between 5,001 and 10,000 nucleotides in length, inclusive. Preferably, the percent sequence identity between the first region of homology in the linear DNA molecule and the corresponding region in the artificial chromosome and the percent sequence identity between the second region of homology in the linear DNA molecule and the corresponding region in the artificial chromosome is at least 90, 92, 94, 96, 98, or 100%. In some embodiments the linear DNA molecule has another region 5' to the first region of homology and/or another region 3' to the second region of homology.

In other embodiments, the first and second regions of the endogenous chromosome are contiguous. In particular embodiments, the first and second regions of the endogenous chromosome are part of the same exon or the same promoter. In other embodiments, the first and second regions of the endogenous

chromosome are not contiguous (e.g., the first and second regions of the endogenous chromosome are part of different exons).

In some embodiments, the selectable marker is inserted into an endogenous promoter or coding sequence and reduces the expression or activity of the protein expressed by the nucleic acid. In other embodiments, the cassette (e.g., the first or second region of homology) has a mutation of interest that is incorporated into the endogenous chromosome. Preferably, the integration of the cassette into the genome of the cell reduces the activity of an RNA (e.g., tRNA) or protein encoded by a nucleic acid of interest (e.g., a nucleic acid that includes a region located between or located within the first and second region of the endogenous chromosome that is mutated by integration of the cassette). In preferred embodiments, the amount of functional protein encoded by the nucleic acid of interest decreases by at least 5,10, 25, 50, 75, 90, 95, or 100%.

In various embodiments, the cassette includes a promoter and/or a polyadenylation signal sequence operably linked to the positive selection maker. In other embodiments, the cassette does not contain a promoter and/or a polyadenylation signal sequence, and the cassette integrates into the genome of the cell such that the positive selection maker is operably linked to an endogenous promoter and/or polyadenylation signal sequence for expression of the selection marker in the cell. In some embodiments, the cassette has two positive selection markers; one selection marker for selection of bacteria or yeast cells with the artificial chromosome having the cassette, and another selection marker for subsequent selection of cells in which a region of the cassette from the artificial chromosome has inserted into the genome. In other embodiments, the cassette has one positive selection marker (e.g., a neomycin resistance gene) operably linked to two different promoters, one promoter for expression of the selection marker in bacteria (e.g., pE7) and another promoter for expression of the selection marker in mouse cells such as embryonic stem cells (e.g., PGK).

In other preferred embodiments, the cassette includes a reporter gene, and the cassette is integrated into the genome of the cell such that the reporter gene is operably linked to an endogenous promoter of interest, thereby generating a genetically modified cell that expresses the reporter gene under the control of the promoter of interest. Exemplary reporter genes include nucleic acids encoding chloramphenicol acetyltransferase, firefly luciferase, renilla luciferase, β-galactosidase, secreted alkaline phosphatase, human growth hormone, β-glucuronidase, green fluorescent protein, or red fluorescent protein. In some embodiments, the method further includes administering one or more compounds to the genetically modified cells and selecting a compound that alters the expression of the reporter gene. This selected compound is useful for modulating the activity of the promoter of interest and thus modulating the expression of endogenous nucleic acids operably linked to the promoter.

In still other preferred embodiments, the cassette includes a nucleic acid encoding a detectable protein (e.g., a fluorescent protein or a protein that catalyzes a reaction in which the reactant or product is detectable), and the cassette is integrated into the genome of the cell such that the nucleic acid is operably linked to an endogenous nucleic acid encoding a protein of interest, thereby generating a genetically modified cell that expresses a fusion protein having the detectable protein and the protein of interest or a fragment thereof. In some embodiments, the nucleic acid encoding the detectable protein replaces a portion of the nucleic acid encoding a protein of interest, thereby generating a fusion nucleic acid that encodes a fusion protein that includes the detectable protein and a fragment of the protein of interest. In other embodiments, the nucleic acid encoding the detectable protein is inserted upstream or downstream of the nucleic acid encoding a protein of interest, thereby generating a fusion nucleic acid that encodes a fusion protein that includes the detectable protein and the entire protein of interest. In some embodiments, the method further includes administering one or more compounds to the genetically modified cells and selecting a compound that alters the

expression of the fusion protein. This selected compound is useful for modulating the expression of the protein of interest.

In desirable embodiments, the steps of the method are repeated, thereby generating a genetically modified cell with two or more mutations. The mutations may be in different alleles of the same gene or in different genes. In some embodiments, each cassette has a recombinase signal sequence, thereby generating a genetically modified cell with two recombinase signal sequences. Recombination may occur between the recombinase signal sequences in the cell.

In some embodiments, the recombinase signal sequences are in the same endogenous chromosome of the cell, and recombination between the recombinase signal sequences results in elimination of the DNA between the recombinase signal sequences. In other embodiments, the recombinase signal sequences are in different endogenous chromosomes of the cell, and recombination between the recombinase signal sequences results in chromosomal translocation between the recombinase signal sequences.

In other preferred embodiments of various aspects of the invention, the cell is an adult, fetal, or embryonic cell. Examples of preferred cells include undifferentiated cells such as embryonic cells (*e.g.*, embryonic stem cells or embryonic germ cells) and differentiated or somatic cells such as epithelial cells, neural cells, epidermal cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, B-lymphocytes, T-lymphocytes, erythrocytes, macrophages, monocytes, fibroblasts, and muscle cells. In another preferred embodiment, the cell is from the female reproductive system, such as a mammary gland, ovarian cumulus, granulosa, or oviductal cell. Other preferred cells include germ and placental cells. Preferred cells also include those from any organ, such as the bladder, brain, esophagus, fallopian tube, heart, intestines, gallbladder, kidney, liver, lung, ovaries, pancreas, prostate, spinal cord, spleen, stomach, testes, thymus, thyroid, trachea, ureter, urethra, and uterus.

The genetically modified cells produced by any of the methods of the

invention can be used as donor cells for the production of non-human mammals with one or more desired genetic modifications. In particular, these cells can be used in any standard method for the generation of chimeric or cloned mammals. In some embodiments, a cell is inserted into an embryo (e.g., a blastocyst) or fetus for the generation of a chimeric mammal that contains some cells with the genetic mutation(s) and some cells without the mutation(s). In other embodiments, a cell or a nucleus from the cell is inserted into an oocyte for the generation of a cloned mammal in which most or all of the cells have the genetic mutation(s).

Accordingly, in one such aspect, the invention provides a method of producing a genetically modified non-human mammal (e.g., a mouse) that involves inserting a cell produced by a method of the invention into a non-human embryo under conditions that allow the embryo to develop into a fetus.

In a related aspect, the invention provides another method of producing a genetically modified non-human mammal (e.g., an ungulate). This method involves inserting a cell produced by a method of the invention or a nucleus from the cell into an oocyte. The oocyte or an embryo formed from the oocyte is transferred into the uterus of a host mammal under conditions that allow the oocyte or the embryo to develop into a fetus.

In preferred embodiments of any of the above aspects for generating mammals, the fetus develops into a live offspring. In some embodiments, one or more cells are isolated for the fetus or live offspring, and one or more additional mutations are introduced into the cells. If desired, these cells can be used to generate mammals with these additional modifications. The methods may also involve mating two of the offspring to generate a mammal with a homozygous mutation or a mammal with a mutation in two or more genes.

In some embodiments, the donor cell has two recombinase signal sequences and recombination occurs between the recombinase signal sequences in cells of a predetermined cell type of the fetus or a live offspring formed from the fetus. For example, the donor cell may be genetically modified to encode a recombinase

under the control of a promoter specific for the predetermined cell type such that the recombinase is only expressed and recombination only occurs in that cell type of the fetus or offspring.

The mammals produced by the above methods can be used in various screening assays to identify a candidate compound that modulates the expression of a nucleic acid or protein of interest or that is useful for the treatment or prevention of a disease. For example, a candidate compound can be administered to a mammal genetically modified to express a reporter gene under the control of a promoter of interest or to express a fusion protein that includes a detectable protein and a protein of interest. The expression levels of the reporter gene or fusion protein can be measured to determine if the candidate compound modulates their expression *in vivo*. Such compounds may be useful for the treatment of a disease associated with a nucleic acid operably linked to the promoter of interest or associated with the protein of interest. Additionally, genetically modified mammals having a mutation associated with a disease can be used as animal models for the study of the disease. Compounds can be administered to these animal models to determine whether they ameliorate or prevent a symptom or other physiological effect associated with the disease.

Accordingly, the invention provides a screening method for determining whether a candidate compound modulates the expression of a nucleic acid of interest. This method involves administering a candidate compound to a mammal (e.g., a mammal that has a genetic modification in a nucleic acid of interest or in a promoter operably linked to a nucleic acid of interest) produced by a method of the invention, and measuring the expression of a nucleic acid. The candidate compound is determined to modulate expression of the nucleic acid if the candidate compound causes a change in expression of the nucleic acid. In some embodiments, measuring the expression of the nucleic acid involves measuring the expression of an mRNA corresponding to the nucleic acid or the expression of a protein encoded by the nucleic acid. Preferably, the mammal is genetically

modified to express a reporter gene operably linked to a promoter of interest (e.g., a promoter that modulates the expression of a nucleic acid associated with a disease). In other embodiments, the mammal is genetically modified to express a fusion protein that includes a detectable protein and a protein of interest or a fragment thereof. In desirable embodiments, the protein of interest is associated with a disease. Exemplary candidate compounds include, for example, proteins, synthesized organic molecules, naturally occurring organic molecules, nucleic acid molecules, and components thereof. In some embodiments, the candidate compound is a member of a library of at least 5, 10, 25, 50, 100, 500, or more compounds that are simultaneously administered to the mammal.

In another aspect, the invention features a screening method for determining whether a candidate compound is useful for the treatment, stabilization, or prevention of a disease, disorder, or condition. This method involves administering a candidate compound to a mammal produced by a method of the invention, and measuring one or more symptoms associated with the disease, disorder, or condition. The candidate compound is determined to be useful for the treatment, stabilization, or prevention of the disease, disorder, or condition if the candidate compound reduces, stabilizes, or prevents the symptom. In desirable embodiments, the genetically modified cell used to generate the mammal has a mutation associated with a disease, and the mammal has one or more phenotypes or symptoms associated with the disease. In some embodiments, the mammal has a mutation, chromosomal deletion, or translocation associated with cancer. Exemplary candidate compounds include, for example, proteins, synthesized organic molecules, naturally occurring organic molecules, nucleic acid molecules, and components thereof. In some embodiments, the candidate compound is a member of a library of at least 5, 10, 25, 50, 100, 500, or more compounds that are simultaneously administered to the mammal.

The invention also provides a method for determining whether a nucleic acid of interest is associated with a disease, disorder, or condition. This method

involves measuring one or more symptoms associated with the disease, disorder, or condition in a mammal that has a mutation in a nucleic acid of interest and that is produced by a method of the invention. The nucleic acid is determined to be associated with the disease, disorder, or condition if the symptom differs between the mammal and a control mammal without the mutation. In some embodiments, a mutation is introduced into an endogenous promoter to increase the expression of a nucleic acid of interest. Exemplary nucleic acids include nucleic acids that are thought to promote cancer such as possible oncogenes; genes that enhance cell proliferation, invasion, or metastasis; genes that inhibit apoptosis; and pro-angiogenesis genes.

The genetically modified cells produced using the methods of the invention can also be administered to a mammal for the treatment, stabilization, or prevention of a disease associated with a deficiency of functional cells of a particular cell-type or associated with a mutation that is not present in the genetically modified cells. For example, the cells can be modified to replace a nucleic acid sequence that is associated with a disease with a nucleic acid sequence that is not associated with a disease.

Accordingly, in one aspect, the invention provides a method of treating, stabilizing, or preventing a disease, disorder, or condition in a mammal (e.g., a human). This method involves administering one or more cells (e.g., purified or unpurified mammalian or human cells) produced by a method of the invention to a mammal in an amount sufficient to treat, stabilize, or prevent the disease, disorder, or condition. In some embodiments, the mammal has a mutation associated with a disease that is not present in the administered cells (e.g., cells that have been genetically modified to eliminate a mutation associated with a disease). Preferably, a disease-causing mutation in a regulatory region, promoter, untranslated region, or coding region of a gene in the cells is modified to replace the mutant sequence with a sequence that is not associated with the disease. Examples of mutations that may be rescued using these methods include dominant

or recessive mutations (e.g., mutations in the cystic fibrosis gene or a gene encoding a human clotting factors such as any of factors I to XIII) (Voet and Voet, Biochemistry, John Wiley & Sons, New York, 1990). In other embodiments, the mammal has a deficiency in the number or activity of cells of a certain cell type.

In some embodiments of the therapeutic methods of the invention, the transplanted cells are genetically modified to introduce a mutation in a promoter or regulatory region that increases the expression of an operably linked nucleic acid that encodes a protein that prevents or ameliorates cancer. Cancer related genes that inhibit cancer include, but are not limited to, tumor suppressor genes; genes that inhibit cell proliferation, invasion, or metastasis; genes that promote apoptosis; and anti-angiogenesis genes. Exemplary cancers include prostate cancers, breast cancers, ovarian cancers, pancreatic cancers, gastric cancers, bladder cancers, salivary gland carcinomas, gastrointestinal cancers, lung cancers, colon cancers, melanomas, brain tumors, leukemias, lymphomas, and carcinomas. Benign tumors may also be treated or prevented using the methods and cells of the present invention.

With respect to the therapeutic methods of the invention, it is not intended that the administration of genetically modified cells to a mammal be limited to a particular mode of administration, dosage, or frequency of dosing; the present invention contemplates all modes of administration, including intramuscular, intravenous, intraarticular, intralesional, subcutaneous, or any other route sufficient to provide a dose adequate to prevent or treat a disease. Preferably, the genetically modified cells are administered to the mammal from which the cells are obtained. Alternatively, the cells may be obtained from a different donor mammal of the same or a different genus or species as the recipient mammal. Examples of preferred donor mammals include humans, cows, sheep, big-horn sheep, goats, buffalos, antelopes, oxen, horses, donkeys, mule, deer, elk, caribou, water buffalo, camels, llama, alpaca, rabbits, pigs, mice, rats, guinea pigs, hamsters, dogs, cats, and primates such as monkeys. The cells may be

administered to the mammal in a single dose or multiple doses. When multiple doses are administered, the doses may be separated from one another by, for example, one week, one month, one year, or ten years. One or more immunosuppressive agents, such as cyclosporin, may be administered to inhibit rejection of the transplanted cells. It is to be understood that, for any particular subject, specific dosage regimes should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions. If desired, conventional treatments may be used in combination with the genetically modified cells of the present invention.

In preferred embodiments of any of the various aspects of the invention, the genetically modified cell has a mutation that alters the expression level or activity of one or more mRNA or protein molecules by at least 2, 5, 10, or 20-fold, as measured using standard assays (see, for example, Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley & Sons, New York, 2000).

The cells and oocytes used in these methods may be from the same species, or they may be from different species or genuses. In preferred embodiments, the oocyte is an enucleated or nucleated non-human oocyte. In addition, the genomic DNA of the cloned embryo, fetus, or mammal is preferably substantially identical to that of the donor cell. In other embodiments, the donor cell is inserted into an embryo for the production of a chimeric embryo, fetus, or mammal containing a mixture of (i) cells with DNA substantially identical to that of the genetically modified donor cell and (ii) cells with DNA substantially identical to that of the naturally-occurring cells in the embryo. Preferred non-human mammals and preferred sources of cells include rodents, such as mice and rats. Examples of other preferred mammals and preferred sources of cells include cows, sheep, big-horn sheep, goats, buffalos, antelopes, oxen, horses, donkeys, mule, deer, elk, caribou, water buffalo, camels, llama, alpaca, rabbits, pigs, guinea pigs, hamsters,

and primates such as monkeys. In some embodiments, the mammal is a murine or an ungulate such as a bovine, ovine, porcine, or caprine.

As used herein, by "artificial chromosome" is meant a chromosome or fragment thereof which has an artificial modification such as the addition of a selectable marker, the addition of a cloning site, the deletion of one or more nucleotides, the substitution of one or more nucleotides, or the like. By "bacterial artificial chromosome (BAC)" is meant an artificial chromosome generated from a low copy plasmid capable of faithfully replicating large chromosomal segments. Similarly, by "yeast artificial chromosome (YAC)" is meant an artificial chromosome generated from one or more yeast chromosomes. By "P1-based artificial chromosome (PAC)" is meant an artificial chromosome generated from the latent replicon of phage P1. If desired, two or more artificial chromosomes can be introduced into a cell (e.g., a bacteria or yeast) simultaneously or sequentially using standard methods (see, for example, Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley & Sons, New York, 2000).

By "donor cell" is meant a cell from which genetic material (e.g., the nucleus or the entire cell) is inserted into an oocyte, embryo, or fetus for the generation of a mammal with a genetic modification.

By "purified" is meant separated from other components that naturally accompany it. Typically, a factor or cell is substantially pure when it is at least 50%, by weight, free from proteins, antibodies, naturally-occurring organic molecules, and cells with which it is naturally associated. Preferably, the factor or cell is at least 75%, more preferably, at least 90%, and most preferably, at least 99%, by weight, pure. A substantially pure factor may be obtained by chemical synthesis, separation of the factor from natural sources, or production of the factor in a recombinant host cell that does not naturally produce the factor. Proteins, vesicles, organelles, and cells may be purified by one skilled in the art using standard techniques such as those described by Ausubel *et al.* (Current Protocols in Molecular Biology, John Wiley & Sons, New York, 2000). The factor or cell is

preferably at least 2, 5, or 10 times as pure as the starting material, as measured using polyacrylamide gel electrophoresis, column chromatography, optical density, HPLC analysis, or western analysis (Ausubel *et al.*, *supra*). Preferred methods of purification include immunoprecipitation, column chromatography such as immunoaffinity chromatography, magnetic bead immunoaffinity purification, panning with a plate-bound antibody, and cell sorting.

By "viable offspring" is meant a mammal that survives *ex utero*. Preferably, the mammal is alive for at least one second, one minute, one hour, one day, one week, one month, six months, or one year from the time it exits the maternal host. The mammal does not require the circulatory system of an *in utero* environment for survival.

By "embryo" or "embryonic" is meant a developing cell mass that has not implanted into the uterine membrane of a maternal host. Hence, the term "embryo" may refer to a fertilized oocyte; an oocyte containing a donor nucleus or cell; a pre-blastocyst stage developing cell mass; or any other developing cell mass that is at a stage of development prior to implantation into the uterine membrane of a maternal host and prior to formation of a genital ridge. An embryo may represent multiple stages of cell development. For example, a one cell embryo can be referred to as a zygote; a solid spherical mass of cells resulting from a cleaved embryo can be referred to as a morula, and an embryo having a blastocoel can be referred to as a blastocyst. An "embryonic cell" is a cell isolated from or contained in an embryo.

By "fetus" is meant a developing cell mass that has implanted into the uterine membrane of a maternal host. A fetus may have defining features such as a genital ridge which is easily identified by a person of ordinary skill in the art. A "fetal cell" is any cell isolated from or contained in a fetus.

By "mutation" is meant an alteration in a naturally-occurring or reference nucleic acid sequence, such as an insertion, deletion, frameshift mutation, silent mutation, nonsense mutation, or missense mutation. Preferably, the amino acid

sequence encoded by the nucleic acid sequence has at least one amino acid alteration from a naturally-occurring sequence.

By "substantially identical" is meant having a sequence that is at least 60, 70, 80, 90, or 100% identical to that of another sequence. Sequence identity is typically measured using sequence analysis software with the default parameters specified therein (*e.g.*, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). This software program matches similar sequences by assigning degrees of homology to various substitutions, deletions, and other modifications.

By "treating, stabilizing, or preventing a disease, disorder, or condition" is meant preventing or delaying an initial or subsequent occurrence of a disease, disorder, or condition; increasing the disease-free survival time between the disappearance of a condition and its reoccurrence; stabilizing or reducing an adverse symptom associated with a condition; or inhibiting or stabilizing the progression of a condition. Preferably, at least 20, 40, 60, 80, 90, or 95% of the treated subjects have a complete remission in which all evidence of the disease disappears. In another preferred embodiment, the length of time a patient survives after being diagnosed with a condition and treated with a cell of the invention is at least 20, 40, 60, 80, 100, 200, or even 500% greater than (i) the average amount of time an untreated patient survives or (ii) the average amount of time a patient treated with another therapy survives.

By "treating, stabilizing, or preventing cancer" is meant causing a reduction in the size of a tumor, slowing or preventing an increase in the size of a tumor, increasing the disease-free survival time between the disappearance of a tumor and its reappearance, preventing an initial or subsequent occurrence of a tumor, or reducing or stabilizing an adverse symptom associated with a tumor. In one preferred embodiment, the percent of cancerous cells surviving the treatment is at least 20, 40, 60, 80, or 100% lower than the initial number of cancerous cells, as

measured using any standard assay. Preferably, the decrease in the number of cancerous cells induced by administration of a cell of the invention is at least 2, 5, 10, 20, or 50-fold greater than the decrease in the number of non-cancerous cells. In yet another preferred embodiment, the number of cancerous cells present after administration of a cell is at least 2, 5, 10, 20, or 50-fold lower than the number of cancerous cells present after administration of a vehicle control. Preferably, the methods of the present invention result in a decrease of 20, 40, 60, 80, or 100% in the size of a tumor as determined using standard methods. Preferably, at least 20, 40, 60, 80, 90, or 95% of the treated subjects have a complete remission in which all evidence of the cancer disappears. Preferably, the cancer does not reappear or reappears after at least 5, 10, 15, or 20 years. In another preferred embodiment, the length of time a patient survives after being diagnosed with cancer and treated with a cell of the invention is at least 20, 40, 60, 80, 100, 200, or even 500% greater than (i) the average amount of time an untreated patient survives or (ii) the average amount of time a patient treated with another therapy survives.

The present invention provides a number of advantages related to the cloning of mammals. For example, the methods decrease the time and labor required to generate genetically modified donor cells which can be used in standard methods to generate cloned or chimeric mammals for medical, research, or agricultural applications. In particular, only small regions of homology from a nucleic acid of interest (e.g., regions that are only 50 nucleotides in length) need to be included in the linear DNA molecule for this molecule to homologously recombine with an artificial chromosome to generate a modified an artificial chromosome with a genetic modification of interest. The presence of a large genomic insert in the artificial chromosome, which is homologous (e.g., at least 90, 92, 96, 98, or 100% identical) to a region of an endogenous chromosome in a cell to be modified, significantly increases the efficiency of homologous recombination between the modified artificial chromosome and the endogenous chromosome and thus reduces the number of transfected cells that need to be

screened for the desired homologous recombination event. Due to the relative ease of producing these donor cells, a large number of mammals with different mutations can be generated simultaneously. These methods can be generally applied to introduce any desired genetic modification, such as a knockout modification, conditional knockout modification, knock-in modification, chromosomal deletion, or chromosomal translocation.

Cells that are modified to correct an undesired mutation (e.g., a mutation associated with a disease) can also be rapidly generated for clinical applications. Because the sequence of an endogenous nucleic acid is modified in the cells, these cell transplantation methods may be more effective for long-term expression of the modified nucleic acid than conventional gene therapy techniques, which may be limited by positional effects due to site of integration of a transgene. Moreover, cells that are transplanted into the mammal from which the donor cells were obtained are unlikely to express foreign antigens and thus are unlikely to induce an adverse immune response that results in rejection of the transplanted cells.

Other features and advantages of the invention will be apparent from the following detailed description and from the claims.

Brief Description of the Drawings

The application file contains drawings executed in color (Figs. 1B, 1C, 1E, 4C, 5B, and 6B). Copies of this patent or patent application with color drawings will be provided by the Office upon request and payment of the necessary fee.

Figure 1A is a schematic diagram of a traditional method for generating knockout mice. Figures 1B and 1C are schematic diagrams of vectors pSKY and pBADλredαβ. Figures 1D and 1E are schematic diagrams of an exemplary procedure for generating a modified BAC. Step 1 involves transforming the pBADλredαβ plasmid into BAC host strain and inducing the expression of αβ genes in the process of making electroporation competent cells. Step 2 involves electroporating the linear substrate and selecting for zeocin/chloramphenicol

double resistant colonies. Step 3 involves identifying homologous recombination events by nested PCR, and step 4 involves extracting DNA. Step 5 involves electroporating the mixed DNA into DH10B cells and selecting for chloramphenicol resistant and ampicillin sensitive colonies.

Figure 2A is a schematic diagram of the generation of a *fancg/xrcc9* knockout construct that illustrates the structures of the wild-type gene (WT), the linear substrate (TG), and the mutant gene (MT). The black blocks represent homologous sequences. The open blocks represent exons. Figure 2B are pictures of gels of nested PCR reaction mixtures that show correct integration at 5'and 3' sites and deletion of exons 5 and 6. Figure 2C is a picture of a gel of a restriction digestion of DNA from wild-type (WT) and two modified BACs (1 & 2) by SpeI and EcoRV. Figure 2D is a picture of a DNA blot of the gel in Fig. 2C with the probe indicated in Fig. 2A.

Figure 3A is a schematic diagram of the generation of a *tiap/survine* conditional knockout construct that shows the structures of the wild-type gene (WT), intermediate recombinants (Steps 1 and 2), and the final product (Step 3). Open blocks represent exons. Figure 3B is a picture of a gel verifying homologous recombination by nested PCR. The primers used in each step are indicated on the right.

Figures 4A-4C are schematic diagrams of strategies for screening targeted ES cells. Homologous recombination can be identified by genomic PCR and DNA blotting when using a conventional targeting construct (Fig. 4A), or by competitive PCR and FISH when using an intact BAC (Figs. 4B and 4C). Gray blocks represent small homologous regions. Broken gray blocks represent large homologous regions. Black blocks represent BAC vector sequences. Downward arrows indicate restriction endonuclease cleavage sites. "WT" denotes the wild-type locus; "TG" denotes the targeting construct; and "MT" denotes the mutant locus.

Figures 5A and 5B illustrate the identification of *fancg/xrcc9*-targeted ES cells. Fig. 5A is a picture of a gel showing the PCR analysis of genomic DNA extracted from clones C38, C52, and C68 and from untargeted ES cells. BAC plasmid DNA (BAC) serves as positive control. PCR fragments from the 5' and 3' ends of the BAC vector and the internal controls are indicated on the left. Fig. 5B is a picture of the FISH analysis of cell lines in Fig. 5A.

Figures 6A and 6B illustrate the identification of *dok3/dok-L*-targeted ES cells. Fig. 6A is a picture of a gel showing the PCR analysis of genomic DNA extracted from indicated clones and untargeted ES cells. Fig. 6B is a picture of the FISH analysis of cell lines in Fig. 6A.

Figures 7A and 7B illustrate the generation of knockout mice. Fig. 7A is a picture of gels showing the genotyping of mouse tail genomic DNA by PCR with primers amplifying wild-type (WT) locus and mutant (MT) locus. Fig. 6B is a picture of gels showing the RT-PCR analysis of mRNA extracted from mouse spleenocytes with primers amplifying exons 5 and 6 from *fancg/xrcc9*, exon 2 from *dok3/dok-L*, and exon 2 from *Iκβras1* to detect the presence of wild-type gene transcripts. Murine β2 microglobulin (for *fancg/xrcc9*) and caspase 8 mRNAs (for *dok3/dok-L* and *Iκβras1*) were used as internal controls.

Figure 8A is a schematic diagram of the generation of a *Iκβras1* knockout construct that illustrates the structures of the wild-type gene (WT), the linear substrate (TG), and the mutant gene (MT). The homologous sequences are labeled as 5' arm and 3' arm. The hatched arrows are FRT sites. The open blocks represent exons. Figure 8B is a picture of a gel of nested PCR reaction mixtures that show correct integration at of the 5'and 3'ends of the antibiotic resistance cassette. Figure 8C is a picture of a gel of a restriction digestion of DNA from wild-type (WT) and modified BACs. Figure 8D is a picture of a DNA blot of the gel in Fig. 8C with the probes indicated in Fig. 8A.

Figure 9A is a schematic diagram of the generation of a *dok3* knockout construct that illustrates the structures of the wild-type gene (WT), the linear substrate (TG), and the mutant gene (MT). The homologous sequences are labeled as 5' arm and 3' arm. The hatched arrows are FRT sites. The open blocks represent exons. Figure 9B is a picture of a gel of nested PCR reaction mixtures that show correct integration at of the 5'and 3' ends of the antibiotic resistance cassette. Figure 9C is a picture of a gel of a restriction digestion of DNA from wild-type (WT) and modified BACs. Figure 9D is a picture of a DNA blot of the gel in Fig. 9C with the probe indicated in Fig. 9A.

Figure 10 is a table illustrating the modification of BACs by homologous recombination in *E. coli*.

Detailed Description

To overcome difficulties associated with conventional site-specific modification methods, we have developed a method that uses modified artificial chromosomes (e.g., BACs, PACs, or YACs) that contain a genetic modification of interest as targeting constructs. The large amount of sequence that is homologous between the artificial chromosome and an endogenous chromosome of the targeted cells greatly increases the efficiency of homologous recombination between the region of the artificial chromosome containing the genetic modification of interest and the corresponding region of the endogenous chromosome. For example, murine BACs contain 100-150 kilobases of mouse genomic sequence that is substantially identical to an endogenous chromosome of a targeted mouse cell, such as an embryonic stem (ES) cell.

For these methods, an artificial chromosome containing a genomic insert (e.g., a region of a mouse chromosome) with a nucleic acid of interest is obtained from a commercial source or constructed using standard methods. A desired genetic modification is introduced into the nucleic acid of interest in the artificial chromosome by homologous recombination between the artificial chromosome

and a linear DNA molecule containing a positive selection marker (e.g., a *zeo*^{r}/*neo*^{r} marker) flanked by two regions of homology to the nucleic acid of interest. In some cases, the desired modification is the insertion of the positive selection marker into the nucleic acid of interest in the artificial chromosome by homologous recombination and the inactivation of the nucleic acid by this insertion. In other cases, one or both regions of homology in the linear DNA molecule have a desired mutation that is introduced into the nucleic acid of interest in the artificial chromosome. If the artificial chromosome is a BAC or PAC, the linear DNA molecule and either the BAC or PAC is introduced into bacteria to allow this homologous recombination to occur. If the artificial chromosome is a YAC, then the homologous recombination is performed in yeast. This method requires very little sequence information about the nucleic acid of interest. For example, homologous regions in the linear DNA fragment as short as 50 nucleotides have been shown to recombine with the corresponding regions in the artificial chromosome to generate the desired modified artificial chromosome. The resulting modified artificial chromosome is introduced into one or more cells (e.g., mouse ES cells), and the genetically modified cells in which homologous recombination occurs between the artificial chromosome and corresponding region of an endogenous chromosome are selected.

Genomic PCR and blotting hybridization are two techniques that have been used in traditional methods to identify homologous recombination in ES cells. Genomic PCR is usually only practical when the flanking region is less then 10 kilobases (Fig. 4A). DNA blot analysis is more reliable but is also limited by the availability of appropriate restriction sites and gel resolution when very large fragments are introduced. Hence, neither method can be applied in general when an entire artificial chromosome is used as a targeting construct in the present methods.

Thus, to use an artificial chromosome (e.g., BAC) for accurate gene replacement in ES or other donor cells, we had to find an effective method to

distinguish homologous recombination from random insertion. We solved this problem by determining copy numbers of the target gene locus using fluorescence *in situ* hybridization (FISH) with the entire artificial chromosome (e.g., BAC) as the probe. This approach allows one to visually distinguish random insertion events, which generate a locus for hybridization, from desired orthotopic targeting events, which maintain the two locus signature of the normal diploid cell. We have observed similar FISH resolution from interphase nuclei and from metaphase chromosome spreads.

If desired, to reduce the number of FISH experiments that are performed, competitive PCR can be used to detect the vector sequences attached to both ends of the artificial chromosome (e.g., BAC) as a primary screening method to exclude random integration events. We found that most of the BACs remained intact after electroporation into ES cells. In particular, 50% of the G418 resistant colonies usually contained at least one vector end sequence. These clones all showed an extra signal when tested by FISH indicating that random integration had occurred. Because the vector sequences attached to both ends of the BAC are very small and probably are the first to be lost during degradation, as many as 80% of the clones passing the initial PCR screening test can be false positives when detected by FISH. Nonetheless, the frequency of targeting is very high, and the procedure is quite simple.

Three independent knockout mice strains have been derived in which the ES clones passed the FISH screening test, suggesting the generality of the present strategy. In all cases, several positive clones were obtained from only one 96-well plate of colonies, indicating consistent high homologous recombination efficiency in ES cells, probably due to the extensive homology shared between the BAC and the targeted gene locus. We were able to obtain correctly targeted ES clones for four different genes in less than two months Additionally, less than eight months was required to construct a Fanconi Anemia protein G (FANCG) -containing BAC and to obtain homozygous *fancg* knockout mice. Two other knockout lines,

Dok-3 and Iκβras1, have also been generated using this method.

In summary, we have developed a method for rapidly generating genetically modified mammals. It involves two steps: modifying artificial chromosomes in bacteria or yeast via homologous recombination and using the mutant artificial chromosomes to modify ES cells or other cells. The system can be used to modify any artificial chromosome without the need for special strains or libraries (Yu *et al.*, Proc. Natl. Acad. Sci. U S A 97:5978-83, 2000 and Zhang *et al.*, Nat. Genet. 30:31-9, 2002). Competitive PCR and FISH are sufficient to screen for homologous recombination events in the cells. The procedure can be streamlined and automated for high-throughput site-specific knockouts production. In principle, it allows several genes to be inactivated simultaneously, which is especially attractive for knocking out families of functionally redundant genes. These methods also provide a simpler and easier solution for more sophisticated genomic manipulations, such as conditional knockouts, knock-ins and large-scale chromosomal engineering (Ramirez-Solis *et al.*, Nature 378:720-724, 1995 and Smith et al., Oncogene 2002. 21:4521-4529, 1995).

These methods are described further below.

Overview of Present Methods for Generating Genetically Modified Mice

As described above, the present method includes two steps, the modification of an artificial chromosome (e.g., a BAC, PAC, or YAC) in bacteria or yeast and gene targeting in the cells to be modified. The general procedure for generating a modified BAC is illustrated in Figs. 1D and 1E; similar methods can also be used to generate other modified artificial chromosomes, such as PACs or YACs. In particular, a linear DNA fragment that has an antibiotic resistance gene flanked by two regions of homology to a nucleic acid of interest is transformed into the BAC host. A plasmid that (i) encodes enzymes that mediate homologous recombination between a short linear DNA fragment and a circular plasmid and that (ii) contains a different antibiotic resistance gene than the linear DNA

molecule is also transformed into the BAC host, and the expression of the enzymes is induced. Double resistant colonies are screened for homologous recombination events by nested PCR. The DNA is extracted from these colonies and used to transform other bacteria. Cells containing the BAC but not the plasmid encoding the recombinases are selected based on the antibiotic resistance of the transformed cells. Correct targeting events are further confirmed, if desired, by comparing restriction endonuclease digestion and DNA blotting. The resulting modified BAC is isolated, linearized, and transformed into the cells (e.g., ES cells) to be modified.

Correct targeting is identified using PCR and fluorescence *in situ* hybridization. The principle is illustrated in Figs. 4B and 4C. When the modified BAC is linearized and electroporated into cells (e.g., ES cells), there are two short fragments from the vector attached to both ends. Homologous recombination results in the loss of these sequences, whereas random integration often keeps them intact with the transgene (Figs. 4B and 4C). Genomic DNA is extracted from resistant colonies (i.e., colonies containing the antibiotic resistant gene from the modified BAC) and subjected to competitive PCR using primers specifically amplifying these fragments along with internal controls primers. Colonies containing either of the vector fragments are discarded (Figs. 4B and 4C).

Although competitive PCR can exclude random integration events, whether the remaining colonies have undergone homologous recombination is confirmed by FISH. If the modified BAC replaces one of the wild-type gene loci as desired, there should be no net gain in gene copy number detected by FISH using the BAC as probe. On the contrary, random integration results in an additional hybridization spot (Figs. 4B and 4C).

Generating a targeting construct by modifying a BAC in *E. coli*

For the generation of a linear DNA fragment containing an antibiotic resistance cassette flanked by two regions of homology, a plasmid designated

pSKY was designed to contain a FRT site-flanked dual selection cassette, which includes an E7 promoter-driven zeocin resistant gene (*zeo*^{r}) for positive selection in bacteria and a phosphoglycerate kinase (PGK) promoter-driven *neo*^{r} for positive selection in ES cells.

In particular, pSKY was constructed using a minimum backbone containing the ColE1 replicon and the ampicillin resistant gene from pBluescript (Stratagene). An E7*zeo*^{r} cassette was PCR amplified from pEM7/Zeo (Invitrogen) and ligated 5' to a PGK*neo*^{r} cassette from pGT-N28 (NEB) with the PGK polyA signal replaced with the SV40 polyA signal and then flanked by FRT sites. Translation stop codons in three reading frames were inserted 5' to the E7*zeo*^{r} cassette to terminate any potential translations from upstream. Convenient cloning sites were generated at both 5' and 3' ends. If desired, the *zeo*^{r}/*neo*^{r} cassette can be removed through recombining the flanking FRT sites by the site-specific recombinase Flp (Zhang *et al.*, Nature Genet. 20:123-128, 1998 and Muyrers *et al.*, Nucleic Acids Res. 27:1555-1557, 1999).

A coding region of the gene of interest is identified by PCR amplification of mouse genomic DNA. This region can be as short as, e.g., 130 base pairs. The primer pair amplifying this region (or the PCR product) is used to obtain a mouse genomic BAC clone containing the gene through the screening service provided by GenomeSystems (now a subsidiary of Incyte Pharmaceuticals). Then, a linear DNA fragment is constructed such that two short regions of homologous sequence from the identified coding region are placed on either side of the *zeo*^{r}/*neo*^{r} cassette in plasmid pSKY (Fig. 1B). In particular, this DNA fragment can be generated by cloning the two regions into the 5' and 3' MCS sites, respectively, in vector pSKY followed by NotI/HindIII digestion. For example, homologous DNA fragments were either generated by PCR amplification or by synthesis and cloned into the 5' EcoRV/EcoRI sites and the 3' BamHI/HindIII sites of pSKY. After sequencing confirmation, the plasmids were digested with NotI/HindIII, and the recombination substrates were gel purified using Qiagen gel extraction kit

(Qiagen). Alternatively, the two homologous regions can be tailed to primers amplifying the *zeo*^{r}/*neo*^{r} cassette, and the linear fragment can be obtained by fusion PCR. The homologous regions can be as short as, e.g., 50 nucleotides and can reside on different exons so that the sequences between them are deleted. We have not reached a lower limit on the possible lengths of the regions of homology or an upper limit on the possible lengths separating the two regions.

Because the DH10B bacteria strain hosting the BACs are recombination deficient, two shuttle vectors, pBADλredαβ and pBADλredαβγ, were constructed to express a partial or full-length phage λ*red* gene under the control of arabinose-inducible pBAD promoter (Fig. 1C). The λ *red* gene encodes three subunits and can mediate homologous recombination between a short linear DNA fragment and a circular plasmid (Zhang *et al.*, *supra* and Muyrers *et al.*, *supra*). Plasmid pBADλredαβ was also constructed using a minimum backbone containing the ColE1 replicon and the ampicillin resistant gene from pBluescript (Stratagene). The λ *red*αβ gene was PCR amplified from phage λ DNA and cloned under the pBAD promoter along with the araC regulatory element from pBAD/His A (Invitrogen).

High homologous recombination efficiency was achieved without utilizing the λ*red*γ gene. The λ*red*γ gene encodes Gam, which inhibits the exonuclease activity of the RecBCD complex in *E.coli* and prevents the linear DNA fragment from degradation. It is possible that the endogenous RecBCD exonuclease does not degrade the linear DNA substrate as quickly as previously believed. An alternative explanation is that its activity is inhibited in the process of making electroporation-competent cells (Karoui *et al.*, Nucleic Acids Res. 27:1296-1299, 1999).

When the entire λ*red*γ gene including the γ subunit was used, we obtained 50 fold more double resistant colonies; nevertheless, the homologous recombination efficiency dropped 4 fold. Whether λ*red*γ also promotes random

insertion is not clear, but high fidelity is desirable when manipulating BAC-sized fragments of DNA because of the need to identify and eliminate clones with unwanted gene rearrangements that occurred during targeting.

The pBADλred shuttle plasmid is transformed into BAC-containing DH10B cells and bacteria resistant to both ampicillin and chloramphenicol are made electroporation-competent after arabinose induction (Figs. 1D and 1E). The linear DNA fragment described above is electroporated into these cells, and its integration into the BAC renders the cells resistant to zeocin and chloramphenicol (Fig. 1E). The double resistant colonies are screened for correct targeting events by nested PCR using two primers annealing outside the homology regions (P3, P4) paired with two primers annealing within the *zeo*^{r}/*neo*^{r} cassette (P1, P2). A PCR product of the correct length that is amplified by primers P1 and P3 indicates homologous recombination at the 5' end. Similarly, homologous recombination at the 3' end is verified by PCR using primers P2 and P4. The correctly modified BAC is separated from the pBADλredαβ shuttle plasmid by electroporating the mixed DNA into DH10B cells and selecting for colonies resistant to chloramphenical, but sensitive to ampicillin (Fig. 1D).

To determine whether there are any random rearrangements in addition to the desired targeting, the digestion patterns of the wild-type BAC plasmid and the modified BAC plasmid, which contains additional restriction sites due to introduction of the *Zeo*^{r}/*neo*^{r} cassette, are compared (Fig. 1E). Incorporation of the antibiotic resistance cassette can be further confirmed, if desired, by DNA Blotting using either the 5' or 3' homology region as a probe.

Targeting of the wild-type gene locus in ES cells using the modified BAC

For the genetic modification of ES cells, the modified BAC DNA is digested by NotI, and the genomic insert with two regions of BAC vector sequence left on both ends is electroporated into the cells. Then, G418-resistant colonies are selected. Genomic DNA of these colonies is extracted and first

screened by competitive PCR. Two pairs of primers are designed to amplify the BAC vector DNA left on either end of the linear fragment (Pa & Pb, Pc & Pd, Figs. 4B and 4C). If homologous recombination occurs, these two regions of BAC DNA are eliminated and not amplified. If random integration occurs, these two regions of BAC DNA remain next to the genomic insert and are amplified by the BAC specific primers. Colonies whose genomic DNA contains the BAC vector specific fragments are discarded. A pair of primers amplifying a larger fragment of mouse caspase-8 gene is used as an internal control in the same PCR reaction.

DNA samples which do not contain the BAC vector specific sequences are subject to DNA Blotting using the same restriction endonuleases and either the 5' or 3' region of homology as a probe (Figs. 4B and 4C). Although this step cannot distinguish homologous recombination from random integration, this step is used to eliminate any clones with incorrect digestion patterns (Figs. 4B and 4C). This step can also be used to select the restriction endonucleases and probes which are later used to identify homozygous mutant mice.

A few of the remaining ES clones are analyzed using fluorescent *in situ* hybridization (FISH) with the whole BAC clone as a probe. If the right locus is targeted, the probe detects two copies of the BAC inserts in the metaphase nucleus in the mutant cells as well as in wild-type, control cells in which no BAC was introduced or in which a control BAC without a knockout cassette was introduced. However, if random integration occurs, more than two copies are detected (Figs. 4B and 4C). ES clones passing all three tests are injected into mouse blastocyst to make chimeras.

We were not able to demonstrate that one of the gene loci harbors the 1.5 kilobase *zeo*^{r}/*neo*^{r} positive selection marker from the knockout cassette using two-color FISH with the *zeo*^{r}/*neo*^{r} marker as a second probe. We suspect that the *zeo*^{r}/*neo*^{r} marker is too short to be used as a specific probe for FISH. Although the presence of this cassette was not confirmed by two-color FISH, the neomycin resistance of the cells with the inserted BAC indicates that this marker is present.

Longer positive selection markers may allow two-color FISH to be used to detect the target gene locus and the positive selection marker simultaneously, if desired.

Generation of Several Modified BACs and a Modified PAC

To demonstrate that the present methods can be used to modify a variety of nucleic acids, a BAC containing a gene with a known genomic structure (e.g., *fancg*) and several BACs containing genes with unknown genomic structures were modified with this procedure. As illustrated in Figs. 2A-2D, 3A, 3B, 8A-8D, and 9A-9D, primer Pzeo and Psv were used to anneal to the antibiotic resistance cassette. Primer Pzeo has the following sequence: 5'-GGC CAG GGT GTT GTC CGG CAC C-3'(SEQ ID NO: 1), and primer Psv has the following sequence: 5'-AAG GTT GGG CTT CGG AAT CG-3' (SEQ ID NO: 2). The primers used for amplifying BAC backbone sequences include Pa: 5'-ACA GAT GCG TAA GGA GAA AAT AC-3' (SEQ ID NO: 3), Pb: 5'-CGC CCT ATA GTG AGT CGT ATT AC-3' (SEQ ID NO: 4), Pc: 5'-ATA GTG TCA CCT AAA TAG CTT GG-3' (SEQ ID NO: 5), and Pd: 5'-GGC ACG ACA GGT TTC CCG ACT GG-3' (SEQ ID NO: 6). The primers used for targeting *fancg/xrcc9* include G34: 5'-CCG TCT TCC AGC CAC GGA GCG GG-3' (SEQ ID NO: 9), G35: 5'-GGC GAT ATC TGC CGT TGG TTC TAA-3'(SEQ ID NO: 10), G36: 5'-CCG AAT TCC AGG CTA CTG GAA A-3' (SEQ ID NO: 11), G37: 5'-CCC GGA TCC CAC CTC CTC TCT AGG-3' (SEQ ID NO: 12), G39: 5'-GGC GAA GCT TTC TGA GCC TTT AGT-3' (SEQ ID NO: 13), G40: 5'-TGG CTA AAT TCA CTA AGT G-3' (SEQ ID NO: 14), Gex5F: 5'-CCT CTG AGG ATC TGC TAC TAC TGC-3' (SEQ ID NO: 15), and Gex6R: 5'-GTG TAC ACC TGG ACT AAC ACG GAC-3' (SEQ ID NO: 16). The primers used for targeting *Ikbras1* include Ikbras1-1: 5'-gggatggaagactgtgaaacgc-3' (SEQ ID NO: 17), Ikbras1-2: 5'-gtctttgaacttatcaatctc-3' (SEQ ID NO: 18), Ikbras1-3: 5'-aggtggcaatcgttgtgttagg-3' (SEQ ID NO: 19), and Ikbras1-4: 5'-ctggatttgctctgggctgag-3' (SEQ ID NO: 20). The primers used

for targeting *dok3* include Dok3-1: 5'-gctggcgcaaagtgggctctg-3' (SEQ ID NO: 21), and Dok3-2: 5'-ccgggaaggccagctgacagatg-3' (SEQ ID NO: 22).

For the preparation of electroporation competent cells, a single BAC clone transformed with pBADλredαβ was grown in 5 ml of LB medium (50 μg/ml ampicillin, 12.5 μg/ml chloramphenicol) until reaching an OD_{600} of 0.6 and diluted into 500 ml of LB medium. The culture was induced with 0.1% L-Arabinose at an OD_{600} of 0.2 and harvested when an OD_{600} of 0.6 was reached. Cells were washed once each with 500 ml of ice-cold ddH_2O, 500 ml of ice-cold 0.4 mM HEPES (pH 7.5) solution, and 5 ml ice-cold 15% glycerol. Cell pellet was re-suspended in equal volume of 10% glycerol and stored in –80°C.

For homologous recombination in *E.coli*, a linear DNA fragment (1 to 2 μg) was electroporated into 100 μl of BAC competent cells at 25 μFD, 2.3 kV and 200 Ω with a Bio-Rad Gene Pulser. Cells were immediately re-suspended in 1 ml of LB medium and inoculated at 37°C for 1.5 hours. Transformants were selected on LB plates containing 20 μg/ml zeocin (Invitrogen) and 12.5 μg/ml chloramphenicol (Sigma). Double resistant colonies were screened by standard colony PCR with primers to amplify both the wild-type and mutant locus. The reaction contains 0.2 mM dNTPs, 0.5 μM of primers, and 1 unit of Taq polymerase (Roche) in 20 μl of total volume containing 1x reaction buffer with 1.5 mM $MgCl_2$ (Roche). The cycling conditions were as follows: 94°C for 3 minutes for 1 cycle; 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 45 seconds for 30 cycles; 72°C for 5 minutes for 1 cycle. Reactions were performed on a Stratagene RoboCycler®. Standard restriction digestion and Southern Blotting were performed.

As summarized in Fig. 10, 10 different BACs were modified with an average efficiency of 60% (25% to 100%). The two homologous regions can reside, e.g., in one or two exons and can be as short as, e.g., 50 nucleotides. We have been able to delete up to about 5 kilobases of sequence between the two

homologous regions. If desired, even shorter regions of homology or even greater distances between the homologous regions may also be used.

We have also successfully modified P1 clones with this method. In addition, we have made conditional knockout constructs in three simple steps using a murine P1 clone containing the *tiap/survine* gene. We first targeted the *zeo*^{r}/*neo*^{r} cassette into intron 1 (step 1, Fig. 3A). We then transduced the homologous recombinants into a Flp expression strain, FLP/DK1. P1 clones with the marker removed were isolated (step 2, Fig. 3A). The *zeo*^{r}/*neo*^{r} cassette was re-introduced into intron 3 (step 3, Fig. 3A). Products in each step were verified by nested PCR (Fig. 3B).

Generation of *fancg/xrcc9* Knockout Mice

As described above, the *zeo*^{r}/*neo*^{r} cassette was introduced into a BAC clone containing the murine *fancg/xrcc9* gene. The marker replaced exons 1 to 10 with 480 nucleotides and 260 nucleotides of homologous sequences from exons 1 and 10, respectively (Fig. 2A). Two primers outside the homologous regions (G34 and G40) paired with two primers inside the *zeo*^{r}/*neo*^{r} cassette (Pzeo and Psv) were used to screen double resistant colonies for correct targeting events at the 5' and 3' end, respectively, by nest PCR (Fig. 2B). Among 24 colonies picked, all produced predicted PCR fragments at both ends. In addition, the PCR product amplified from exons 5 and 6 was only observed in the wild type BAC, suggesting these exons were deleted in the modified BACs (Fig. 2B).

To determine whether any unintended rearrangements were generated during modification, the digestion patterns of EcoRV (i.e., a restriction site that is absent in the linear DNA fragment) or SpeI (i.e., a restriction site that is unique in the linear DNA fragment) restriction sites of two independent modified BACs were compared with those of the wild-type BAC. No gross rearrangements were detected in the modified BACs. (Fig. 2C). DNA blotting using the 5' homologous region as probe demonstrated that there was a single copy of the marker integrated

at the desired site (Fig. 2D). The fidelity of this procedure was further confirmed by shotgun sequencing of the modified BAC.

This modified BAC was used to target the *fancg/xrcc9* locus in ES cells. In particular, BAC DNA was purified using a Qiagen Large-Construct kit, linearized by NotI digestion, extracted once using phenol:$CHCl_3$, precipitated, and resuspended in 0.1x TE buffer (pH 8.0) at 1 μg/μl. ES cells were cultured according to standard conditions (DePamphilis, ed. Methods in Enzymology, Vol. 225 Academic press, Inc., 1993), and $6x10^6$ ES cells were electroporated with 30 μg of BAC DNA at 0.23 kV, 960 μFD by a Bio-Rad Gene Pulser. Selection was started the second day after transfection with medium containing 400 μg/ml G418 (Invitrogen/Gibco). Colonies were picked into 96-well plates. Genomic DNA was extracted according to standard protocols (DePamphilis, *supra*). About 0.1 μg of genomic DNA was used as templates in competitive genomic PCR. Cycling conditions were the same as described above. The control primers for competitive PCR include forward: 5'-GAG GAC ATC TTT CCC TCA GGC-3' (SEQ ID NO: 7), and reverse: 5'-CAG AGG CTC TGA GTA AGA CC-3' (SEQ ID NO: 8).

Among 96 PCR reactions, 38 showed both the control PCR fragment and the BAC vector fragment (e.g., C68 in Fig. 5A), 22 showed only the control PCR fragment (e.g., C38 and C52, Fig. 5A) and 36 showed neither. We performed FISH on clones C38, C52, and C68 along with the wild type ES cells using the entire BAC as probe. For this analysis, standard FISH procedures were applied with minor modifications. For staining interphase nuclei, about 10^5 cells were either directly seeded on a 10-well slide (Fisher) or on a regular slide (Fisher) by spinning in a Cytospin centrifuge. Slides were air-dried briefly and fixed in freshly made 4% paraformadehyde in PBS (pH 7.4) for 10 minutes at room temperature. After rinsing off the excess fixative in PBS, the slides were stored in 70% ethanol at 4°C. For staining metaphase spread chromosomes, the cells were treated, and the slides were prepared according to standard procedures. Either

random priming (Prime-It® Fluor Fluorescence Labeling Kit, Stratagene) or nick-translation (BioNick DNA labeling system, Invitrogen/Gibco) worked well for labeling BACs. Under the condition we used, random priming yielded stronger signals. Manufacturers' protocols were followed and 35 μg COT-1 DNA (Invitrogen/Gibco) were usually used to suppress nonspecific signals. The probe was dissolved in Hybrisol VI (Ventana) and then denatured at 75°C for 10 minutes followed by incubation at 42°C for 1 hour. Slides were dehydrated two minutes each in 80%, 85%, 95%, and 100% ethanol. After air-drying, slides were denatured in 70% deionized formamide (American Bioanalytical), 2x SSC (pH 7.0) at 72°C for 10 minutes and dehydrated through ice-cold 70%, 80%, 95%, and 100% ethanol. The probe was adjusted to 10 ng/μl, and 4 μl was applied per sample area. Hybridization was carried overnight in a humid chamber at 42°C. Slides were washed three times in 2x SSC (pH 7.4) at 72°C for 7 minutes each and once in 0.2xSSC (pH 7.4) at 72°C for 7 minutes. Signals were then amplified using TSA™ Fluorescence Systems (NEN™ Life Science Products) following the manufacturer's protocol. Slides were finally counterstained with DAPI in 4x SSC, mounted in Vectashield (Vector Laboratories, Inc.), and observed under a Zeiss Axioplan 2 fluorescence microscope.

Using this FISH analysis, we detected two signals in clones C38 and C52 as well as in the untargeted ES cells (Fig. 5B) and an extra signal in clone C68 (Fig. 5B), suggesting that C38 and C52 were correct targeting products and C68 was the result of random integration.

To confirm that the ES clones identified by FISH represented correct targeting events, several ES clones were microinjected into C57BL6 blastocysts, and mice were bred to homozygosity according to standard procedures (DePamphilis, *supra*). For PCR genotyping, mouse tail genomic DNA was extracted according to standard protocols (DePamphilis, *supra*), and PCR reactions were performed as described for competitive PCR. We obtained one germline-transmission competent chimera for *fancg/xrcc9*. The resulting mice had

homozygous mutations, as indicated by genomic PCR genotyping (Figs. 7A and 7B). Phenotype analysis of *fancg/xrcc9* deficient mice is described by Yang *et al.*, (Blood 98:3435-3440, 2001).

Generation of *Iκβras1* and *dok-3/dok-L* Knockout Mice

BACs containing mutations in *Iκβras1* or *dok-3/dok-L* were also used to modify mouse ES cells as described above (Figs. 8A-8D and 9A-9D). We found that the PCR data did not always correlate with the FISH data. For example, in targeting the *dok-3/dok-L* gene, we discovered that clone C10 did not contain BAC vector sequences as judged by PCR (Fig. 6A) but had three signals as observed by FISH (Fig. 6B). These results suggest that FISH can determine true gene replacement events while competitive PCR only serves as a primary screening method to reduce the number of FISH experiments to be conducted.

Several ES clones identified by FISH were injected into mice blastocysts and bred to produce homozygous mice. We obtained one germline-transmission competent chimera each for *Iκβras1* and *dok-3/dok-L*. Both chimeras resulted in mice with homozygous mutations as indicated by genomic PCR genotyping (Figs. 7A and 7B).

Generation of Conditional Knockouts, Knock-ins, and Chromosomal Alterations

In desired, a cell can be genetically modified such that a deletion mutation only occurs in certain cells of a mammal generated using the genetically modified donor cell. In particular, the donor cell has two recombinase signal sequences (e.g., recombinase signal sequences within introns that flank one or more exons to be deleted) and recombination occurs between the recombinase signal sequences in cells of a predetermined cell type of the resulting mammal. For example, the donor cell may be genetically modified to encode a recombinase under the control of a promoter specific for the predetermined cell type such that the recombinase is only expressed and recombination only occurs in that cell type of mammal.

Alternatively, a mammal modified to contain the recombinase signal sequences can be mated with a mammal modified to express the recombinase under the control of a promoter specific for the predetermined cell type.

Knock-in mutations can be generated by including a nucleic acid such as a reporter gene or nucleic acid encoding a detectable protein between the regions of homology in the linear DNA fragment used to generate the modified BAC. Desirably, the reporter gene is integrated under the control of an endogenous promoter of interest. The nucleic acid encoding a detectable protein is desirably integrated in-frame with an endogenous nucleic acid encoding a protein of interest for the generation of a fusion nucleic acid the encodes a fusion protein with the detectable protein and the protein of interest.

For the generation of chromosomal alterations, a genetically modified cell is generated with two recombinase signal sequences. If the recombinase signal sequences are in the same endogenous chromosome of the cell, recombination eliminates the DNA between the recombinase signal sequences. If the recombinase signal sequences are in different endogenous chromosome of the cell, recombination results in chromosomal translocation between the recombinase signal sequences. Chromosome deletions can also be generated by using a linear DNA molecule that has two regions of homology that correspond to regions of the endogenous chromosome that flank the DNA to be deleted (e.g., a single gene or a cluster of genes to be deleted). The cells and mammals generated using this linear DNA molecule have the desired deletion mutation. If desired, this method can be repeated to delete an additional region beside the first deletion site.

Optional Modifications to Further Increase the Efficiency of the Present Methods

To reduce the time required to generate bacteria containing both a BAC and the pBADλredαβ plasmid, the pBADλredαβ plasmid can be transferred to bacteria containing the BAC by conjugation. In particular, we have discovered that plasmids containing either the OriT region from F' or the bom-mob complex

from the ColE1 plasmid can by mobilized by a male *E. coli* strain (e.g., XL-1Blue, an F'-bearing strain) to the BAC host strain DH10B through conjugation. In both cases, the small plasmid is preferentially transferred relative to the mobilizing F'. To further reduce the contribution of episomal elements, the small plasmids can be introduced into an Hfr strain of *E. coli*. We have generated plasmids that contain oriT in the pBADλredαβ plasmid or that contain bom-mob of ColE1, the p15A replicon from plasmid pACYC184, and λred αβ or λred αβ plus PI-SceI under the control of either the Ara BAD promoter or the lac uv5 promoter. These plasmids can be introduced into the BAC host by growing the two strains together for one hour and selecting for ampicillin and chloramphenicol double resistant colonies, or kanamycin and chloramphenicol resistant colonies, depending on the resistance of the BAC plasmid.

If desired, a temperature sensitive replicon can be used so that cells containing only the modified BAC can be isolated by killing cells containing the pBADλredαβ plasmid by shifting the cultures to a higher temperature. In particular, the ColE1 replicon on the pBADλredαβ plasmid can be replaced with the temperature sensitive (ts) replicon from pSC101. Cells with this pBADλredαβ plasmid are unable to grow at 42°C, so elevated temperatures can be used to separate this plasmid from the modified BAC.

If desired, the linear DNA molecule used for generation of the modified BAC can be generated *in vivo* by cleavage of a circular fragment containing the sequence of the linear DNA fragment flanked by SceI sites. The VDE/PI-SceI system can be used according to the manufacturer's instructions for this procedure. Thus, a bacterial strain containing a BAC, a bacterial strain containing the pBADλredαβ plasmid, and a bacterial plasmid with the sequence of the linear DNA fragment flanked by SceI sites can be grown together, and the BAC and the two plasmids can be transferred into the same bacteria by conjugation and antibiotic selection. This method may eliminate the need for electroporation of

competent cells and allow library screening and BAC modification to be performed in a single step.

Generation of Other Genetically Modified Mammals

The methods described above for the genetic modification of mice can generally be applied to the genetic modification of any non-human mammal. In particular, an artificial chromosome (e.g., a BAC) containing a genomic insert from another mammal can be purchased or constructed using standard methods (Ausubel *et al.*, *supra*). The BAC can be homologously recombined with a linear DNA molecule containing a positive selection marker flanked by two regions with homology to a nucleic acid in the BAC as described above. The resulting modified BAC can be used to alter the genome of cells from the same genus or species as the source of the genomic insert in the BAC. A variety of cells can be modified including embryonic, fetal, and adult differentiated or undifferentiated cells. The resulting modified cells can be used in any standard method for the generation of a cloned or chimeric mammal. For example, genetically modified ES cells can be injected into an embryo for the generation of chimeric mammals as described above. Alternatively, an embryonic or somatic cell or a nucleus from the cell (e.g., an ungulate cell) can be inserted into an oocyte (e.g., an enucleated oocyte). After activation of the oocyte, the oocyte is placed in culture medium for an appropriate amount of time to allow development of the resulting embryo. At the two cell stage or a later stage, the embryo is transferred into a foster recipient female for development to term (see, for example, U.S. Patent Numbers 4,994,384; 6,077,710; 5,453,366; 5,945,577; 6,258,998; and 5,057,420; Wakayama *et al.*, PNAS 96:14984-14989, 1999; Wakayama *et al.*, Nature Genetics 24:108-109, 2000; and Stice and Keefer, Biology of Reproduction 48: 715-719, 1993).

Exemplary Applications of the Present Methods

The mammals produced by any of the methods of the present invention can be used as animal models to identify candidate compounds that modulate the expression of a nucleic acid or protein of interest or that are useful for the treatment or prevention of a disease. For example, a mammal can be genetically modified to express a reporter gene under the control of a promoter of interest by the integration of a knock-in cassette containing a reporter gene that is flanked by a region homologous to the promoter of interest and another region of homology. Candidate compounds are administered to this mammal to determine whether they modulate the activity of the promoter of interest and thus are useful for the treatment of a disease associated with a nucleic acid operably linked to the promoter of interest. Alternatively, a mammal can be genetically modified to express a fusion protein that includes a detectable protein and a protein of interest. This mammal is generated, e.g., by the integration of a knock-in cassette containing two regions homologous to a nucleic acid of interest (i.e., the nucleic acid encoding the protein of interest) flanking a nucleic acid encoding the detectable protein. Candidate compounds are administered to this mammal to determine if they modulate the expression of this fusion protein *in vivo*. Such compounds may be useful for the treatment of a disease associated with the protein of interest.

The present methods can also be used to generate animal models of various diseases. In particular, one or more mutations associated with a disease can be introduced into a mammal (e.g., a mouse, ungulate, or primate) as described herein. Candidate compounds can be administered to these mammals to determine whether the compounds ameliorate or prevent a symptom or other physiological effect associated with the disease. Exemplary mutations associated with cancer or diabetes are described by Ruediger *et al.* (Oncogene 20:10-15, 2001) and Yokoi *et al.* (Nature Genetics 31:391-394, 2002), respectively. Chromosomal translocations associated with cancer (e.g., acute myelogenous leukemia) are also

described by the following references: Kuehl *et al.*, Nature Reviews Cancer 2(3):175-87, 2002; Dyer *et al.*, Leukemia 16(6):973-84, 2002; Gojo *et al.*, Cancer Treatment & Research 108:231-55, 2001; Padua *et al.*, Cancer Treatment & Research 108:111-57, 2001; Falini *et al.*, Blood 99(2):409-26, 2002; Stevenson *et al.*, Advances in Cancer Research 83:81-116, 2001; Swerdlow *et al.*, Human Pathology 33(1):7-20, 2002; Chakraborty *et al.*, Journal of Cellular Biochemistry 82(2):310-25, 2001; Kelly *et al.*, Current Opinion in Oncology 14(1):10-8, 2002; Falini *et al.*, British Journal of Haematology 114(4):741-60, 2001; Davila *et al.*, Advances in Cancer Research 81:61-92, 2001; Sturzl *et al.*, Advances in Cancer Research 81:125-59, 2001; Arvand *et al.*, Oncogene 20(40):5747-54, 2001; Barr *et al.*, Oncogene 20(40):5736-46, 2001; Seidel *et al.*, Oncogene 20(40):5718-25, 2001; Aspland *et al.*, Oncogene 20(40):5708-17, 2001; Alcalay *et al.*, Oncogene 20(40):5680-94, 2001; Licht *et al.*, Oncogene 20(40):5660-79, 2001; Duyster *et al.*, Oncogene 20(40):5623-37, 2001; Bergsagel *et al.*, Oncogene 20(40):5611-22, 2001; Boxer *et al.*, Oncogene 20(40):5595-610, 2001; Kuppers *et al.*, Oncogene 20(40):5580-94, 2001; Mullauer *et al.*, Mutation Research 488(3):211-31, 2001; Morris *et al.*, British Journal of Haematology 113(2):275-95, 2001; Lamorte *et al.*, Surgical Oncology Clinics of North America 10(2):271-88, viii, 2001; Maru, International Journal of Hematology 73(3):308-22, 2001; Sattler *et al.*, International Journal of Hematology 73(3):278-91, 2001; Holyoake, British Journal of Haematology 113(1):11-23, 2001; Bower, British Journal of Haematology 112(4):863-73, 2001; Crans *et al.*, Leukemia 15(3):313-31, 2001; Lee, British Journal of Haematology 111(4):993-1009, 2000; Mavrothalassitis *et al.*, Oncogene 19(55):6524-32, 2000; Garcia-Manero *et al.*, Hematology - Oncology Clinics of North America 14(6):1381-96, x-xi, 2000; Larson, Hematology - Oncology Clinics of North America 14(6):1367-79, x, 2000; Thiebaut *et al.*, Hematology - Oncology Clinics of North America 14(6):1353-66, x, 2000; Durrant *et al.*, Hematology - Oncology Clinics of North America 14(6):1327-52, 2000; Faderl *et al.*, Hematology - Oncology Clinics of North

America 14(6):1267-88, 2000; Drexler *et al.*, Leukemia 14(9):1533-59, 2000; Pui *et al.*, British Journal of Haematology 109(1):13-23, 2000; and Dierlamm *et al.*, Hematological Oncology 18(1):1-13, 2000.

The genetically modified mammals of the invention are also useful for target validation to confirm that a nucleic acid of interest is associated with a disease, disorder, or condition. For example, one or more symptoms associated with the disease, disorder, or condition can be compared between a mammal having a mutation in the nucleic acid of interest or having a mutation that alters the expression of a nucleic acid of interest (e.g., a mutation in a promoter or the insertion of a exogenous promoter) and a control mammal without the mutation. Exemplary nucleic acids include nucleic acids that are thought to promote cancer such as possible oncogenes; genes that enhance cell proliferation, invasion, or metastasis; genes that inhibit apoptosis; and pro-angiogenesis genes.

Moreover, the present methods can be used as an alternative to conventional gene therapy methods to correct a mutation associated with a disease in cells from a patient (e.g., a human) that are subsequently readministered to the patient or administered to another patient (Kyba *et al.*, Cell. 109(1):29-37 2002; Rideout *et al.*, Cell. 109(1):17-27, 2002). In these applications, cells (e.g., cells from a human patient such as primary cells, bone marrow cells, or blood cells) are genetically modified to replace a region of nucleic acid that has one or more mutations associated with a disease with a nucleic acid segment without the mutation(s). This genetic modification procedure can be repeated, if desired, to correct both alleles of the nucleic acid. Then, standard methods can be used to administer the modified cells into the appropriate area of the patient.

Other Embodiments

From the foregoing description, it will be apparent that variations and modifications may be made to the invention described herein to adopt it to various

usages and conditions. Such embodiments are also within the scope of the following claims.

All publications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.

What is claimed is:

Claims

1. A method of producing a genetically modified cell, said method comprising the steps of:

(a) inserting into one or more cells an artificial chromosome comprising a cassette which includes a first region of homology having substantial sequence identity to a first region of an endogenous chromosome of said cell(s), a selectable marker, and a second region of homology having substantial sequence identity to a second region of said endogenous chromosome under conditions that result in homologous recombination between said artificial chromosome and said endogenous chromosome and integration of said cassette into said endogenous chromosome of one or more cells; and

(b) selecting a cell in which said homologous recombination occurs, thereby selecting a genetically modified cell.

2. The method of claim 1, wherein said artificial chromosome comprising said cassette is produced by a method comprising the steps of:

(a) culturing a cell that has (i) a linear DNA molecule comprising said cassette and (ii) an artificial chromosome comprising nucleic acid that is substantially identical to said first and second regions of homology under conditions that result in homologous recombination between said linear DNA molecule and said artificial chromosome, thereby generating said artificial chromosome comprising said cassette.

3. The method of claim 2, wherein said linear DNA molecule is introduced into said cell by transformation.

4. The method of claim 2, wherein said linear DNA molecule is introduced into said cell by insertion of a circular vector comprising the sequence of said

linear DNA molecule into said cell and cleavage of said vector to generate said linear DNA molecule inside said cell.

5. The method of claim 1, wherein said first and second regions of said endogenous chromosome are contiguous.

6. The method of claim 5, wherein said first and second regions of said endogenous chromosome are part of the same exon or the same promoter.

7. The method of claim 1, wherein said first and second regions of said endogenous chromosome are not contiguous.

8. The method of claim 7, wherein said first and second regions of said endogenous chromosome are part of different exons.

9. The method of claim 1, wherein the integration of said cassette into the genome of said cell reduces the activity of the protein encoded by a nucleic acid of interest.

10. The method of claim 1, wherein the amount of functional protein encoded by said nucleic acid of interest decreases by at least 25%.

11. The method of claim 1, wherein said cassette comprises a reporter gene, and wherein said cassette is integrated into the genome of said cell such that said reporter gene is operably linked to an endogenous promoter of interest, thereby generating a genetically modified cell that expresses said reporter gene under the control of said promoter.

12. The method of claim 1, wherein said cassette comprises a nucleic acid encoding a detectable protein, and wherein said cassette is integrated into the genome of said cell such that said nucleic acid is operably linked to an endogenous nucleic acid encoding a protein of interest, thereby generating a genetically modified cell that expresses a fusion protein comprising said detectable protein and protein of interest or fragment thereof.

13. The method of claim 1, further comprising repeating steps (a) and (b), thereby generating a genetically modified cell with two or more mutations.

14. The method of claim 13, wherein each cassette comprises a recombinase signal sequence, thereby generating a genetically modified cell with two recombinase signal sequences.

15. The method of claim 14, wherein recombination occurs between said recombinase signal sequences in said cell.

16. The method of claim 15, wherein said recombinase signal sequences are in the same endogenous chromosome of said cell, and wherein recombination between said recombinase signal sequences results in elimination of the DNA between said recombinase signal sequences.

17. The method of claim 15, wherein said recombinase signal sequences are in different endogenous chromosome of said cell, and wherein recombination between said recombinase signal sequences results in chromosomal translocation between said recombinase signal sequences.

18. The method of claim 1, wherein said cell is an embryonic stem cell.

19. The method of claim 1, wherein said cell is a somatic cell.

20. A method of producing a genetically modified non-human mammal, said method comprising inserting a cell produced by the method of claim 1 into a non-human embryo under conditions that allow said embryo to develop into a fetus.

21. A method of producing a genetically modified non-human mammal, said method comprising the steps of:

(a) inserting a cell produced by the method of claim 1 or a nucleus from said cell into an oocyte; and

(b) transferring said oocyte or an embryo formed from said oocyte into the uterus of a host mammal under conditions that allow said oocyte or said embryo to develop into a fetus.

22. The method of claim 20 or 21, wherein said fetus develops into a live offspring.

23. The method of claim 22, further comprising mating two of said offspring to generate a mammal with a homozygous mutation.

24. The method of claim 22, further comprising mating two of said offspring to generate a mammal with a mutation in two or more genes.

25. The method of claim 20 or 21, wherein said cell comprises two recombinase signal sequences and recombination occurs between said recombinase signal sequences in cells of a predetermined cell type of said fetus or a live offspring formed from said fetus.

26. The method of claim 20 or 21, wherein said cell is an embryonic stem cell.

27. The method of claim 20 or 21, wherein said cell is a somatic cell.

28. The method of claim 20 or 21, wherein said mammal is a murine, bovine, ovine, porcine, or caprine.

29. The method of claim 28, wherein said mammal is a murine.

30. A screening method for determining whether a candidate compound modulates the expression of nucleic acid of interest, said method comprising the steps:

(a) administering a candidate compound to a mammal that has a genetic modification in a nucleic acid of interest or in a promoter operably linked to a nucleic acid of interest and that is produced by the method of claim 20 or 21; and

(b) measuring expression of a nucleic acid of interest, whereby said candidate compound is determined to modulate expression of said nucleic acid if said candidate compound causes a change in expression of said nucleic acid.

31. The method of claim 30, wherein step (b) comprises measuring the expression of an mRNA corresponding to said nucleic acid.

32. The method of claim 30, wherein step (b) comprises measuring the expression of a protein encoded by said nucleic acid.

33. The method of claim 30, wherein said mammal is genetically modified to express a reporter gene operably linked to a promoter of interest.

34. The method of claim 30, wherein said mammal is genetically modified to express a fusion protein comprising a detectable protein and a protein of interest or a fragment thereof.

35. A screening method for determining whether a candidate compound is useful for the treatment, stabilization, or prevention of a disease, disorder, or condition, said method comprising the steps:

(a) administering a candidate compound to a mammal produced by the method of claim 20 or 21; and

(b) measuring one or more symptoms associated with a disease, disorder, or condition, whereby said candidate compound is determined to be useful for the treatment, stabilization, or prevention of said disease, disorder, or condition, if said candidate compound reduces, stabilizes, or prevents said symptom.

36. The method of claim 35, wherein said mammal has a chromosomal deletion or translocation associated with cancer.

37. A method for determining whether a nucleic acid is associated with a disease, disorder, or condition, said method comprising measuring one or more symptoms associated with a disease, disorder, or condition in a mammal that has a mutation in a nucleic acid of interest and that is produced by the method of claim 20 or 21, whereby said nucleic acid is determined to be associated with said disease, disorder, or condition if said symptom differs between said mammal and a control mammal without said mutation.

38. A method of treating, stabilizing, or preventing a disease, disorder, or condition in a mammal, said method comprising administering one or more cells produced by the method of claim 1 to a mammal in an amount sufficient to treat, stabilize, or prevent said disease, disorder, or condition.

5

39. The method of claim 38, wherein said mammal is a human.

METHODS FOR THE PRODUCTION OF CELLS AND MAMMALS WITH DESIRED GENETIC MODIFICATIONS

Abstract of the Disclosure

The present invention features novel methods for generating cell lines and mammals with site-specific genetic modifications of interest. The methods involve homologous recombination between an artificial chromosome having a modification of interest and an endogenous chromosome of a cell. The resulting modified cells can be used in standard methods to generate genetically modified mammals. These mammals can be used in a variety of screening methods to identify compounds useful for the treatment or prevention of disease. Additionally, cells that have been modified to eliminate a mutation associated with a disease can be transplanted into patients for the treatment of a disease.

PATENT
ATTORNEY DOCKET NO. 00786/432001

Certificate of Mailing

Date of Deposit August 2, 2002 Label Number EL 947117528 US

I hereby certify under 37 C F.R § 1.10 that this correspondence is being deposited with the United States Postal Service as "Express Mail Post Office to Addressee" with sufficient postage on the date indicated above and is addressed to BOX PROVISIONAL APPLICATION, Assistant Commissioner for Patents, Washington, D.C. 20231.

Guy E. Beardsley
Printed name of person mailing correspondence

Signature of person mailing correspondence

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Brian Seed et al.	Art Unit:	Not yet assigned
Serial No.:	Not yet assigned	Examiner:	Not yet assigned
Filed:	August 2, 2002	Customer No.:	21559
Title:	METHODS FOR THE PRODUCTION OF CELLS AND MAMMALS WITH DESIRED GENETIC MODIFICATIONS		

Assistant Commissioner For Patents
Washington, DC 20231

PETITION TO ACCEPT COLOR DRAWINGS

Applicants hereby petition under 37 C.F.R. § 1.84(a)(2), that the enclosed color drawings are necessary as the only practical medium to disclose the subject matter of the present application. The subject matter of the invention relates to methods for genetically altering cells and mammals. The color drawings in Figures 1B, 1C, 1E, 4C, 5B, and 6B are necessary to accurately and clearly depict examples of the subject matter sought to be patented.

Enclosed is a check for $130.00 to cover the fee required under 37 C.F.R. § 1.17(h) and three sets of the color drawings. Applicants have also included black and

08/07/2002 HTECKLU1 00000029 60400900
02 FC:122 130.00 OP

white versions of these drawings as part of the application.

As required under 37 C.F.R. § 1.84(a)(2)(iv), the first paragraph of the Brief Description of the Drawings States that:

> The application file contains drawings executed in color (Figs. 1B, 1C, 1E, 4C, 5B, and 6B). Copies of this patent or patent application with color drawings will be provided by the Office upon request and payment of the necessary fee.

If there are any charges, or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Date: August 2, 2002

Vicki L. Healy

Karen L. Elbing, Ph.D. Vicki L. Healy
Reg. No. 35,238 Reg. No. 48,343

Clark & Elbing LLP
101 Federal Street
Boston, MA 02110
Telephone: 617-428-0200
Facsimile: 617-428-7045

\\Clark-w2k1\documents\00786\432xxx\00786.432001 Petition to Accept Color Drawings.wpd

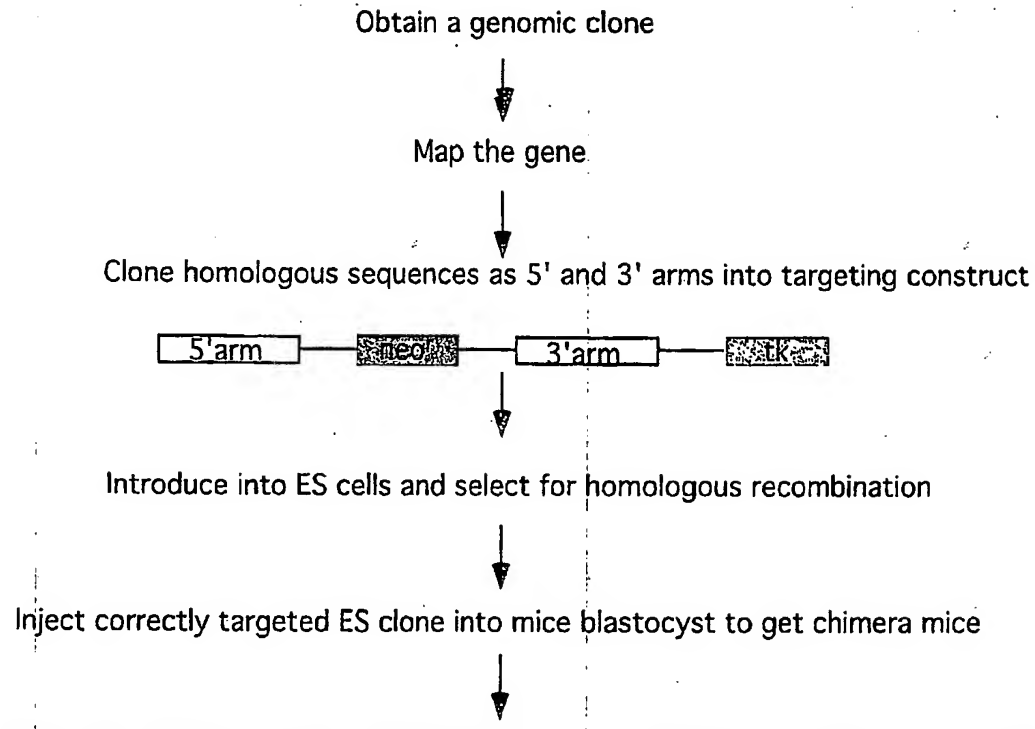
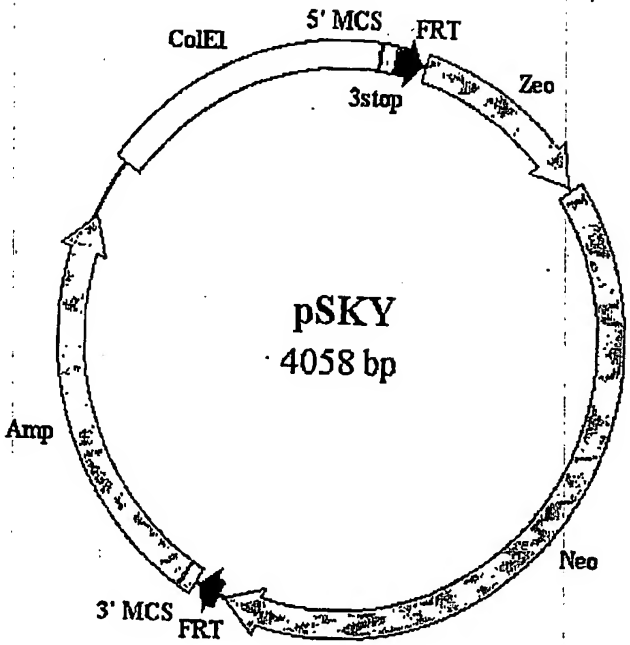
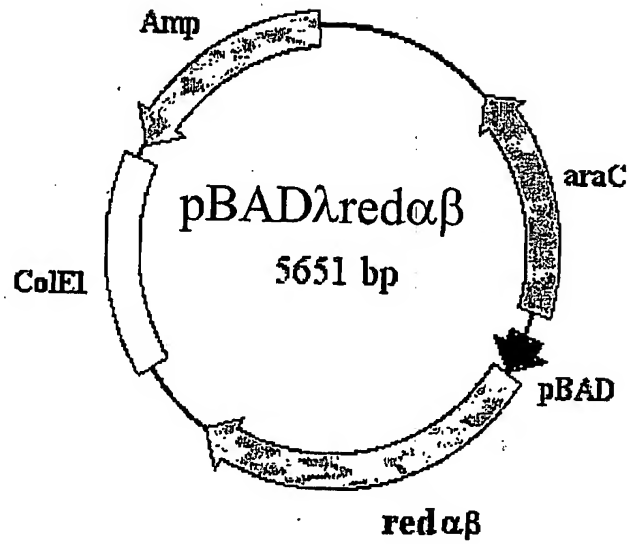


Figure 1A

B



C



Figures 1B and 1C

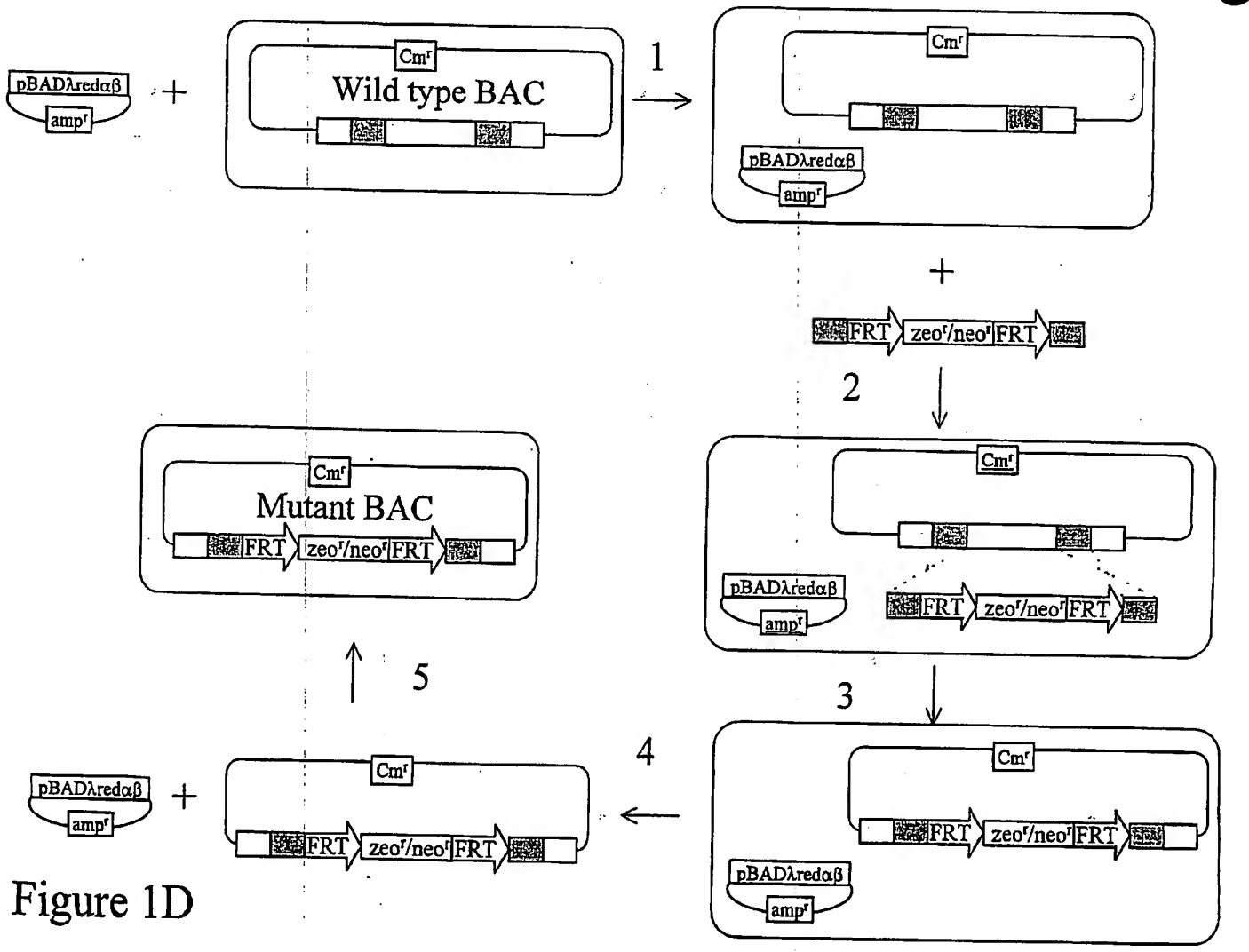


Figure 1D

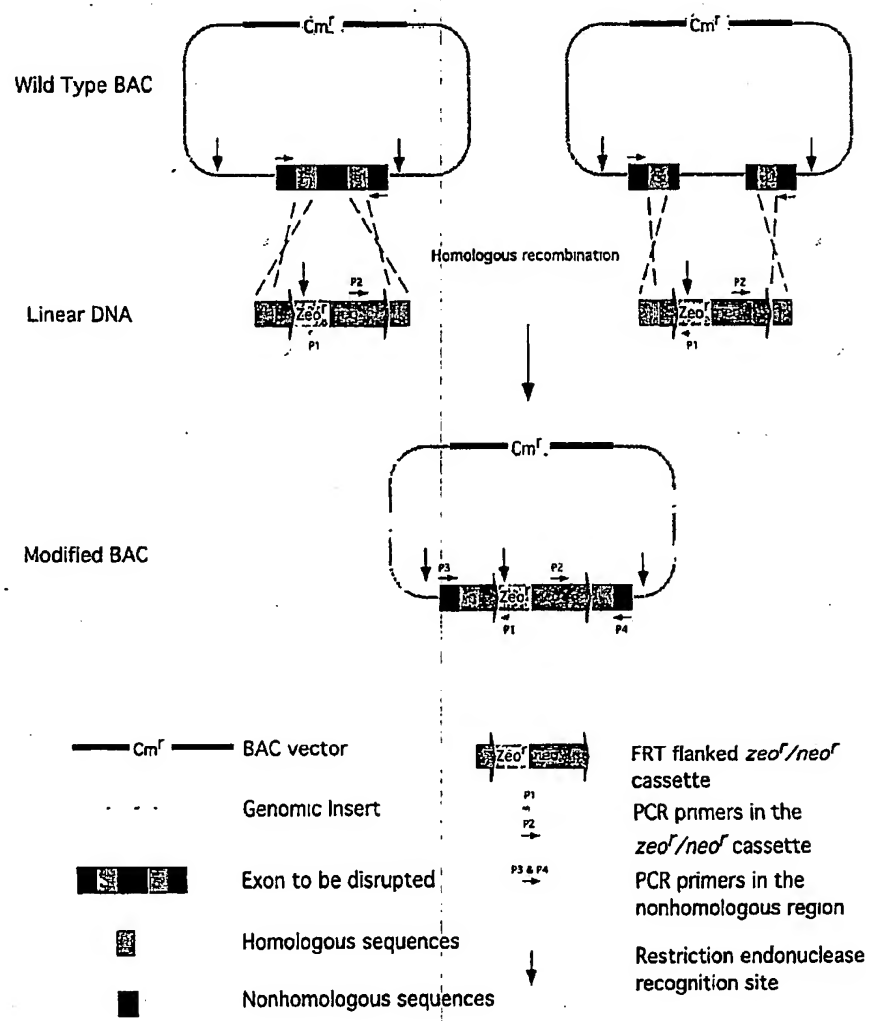
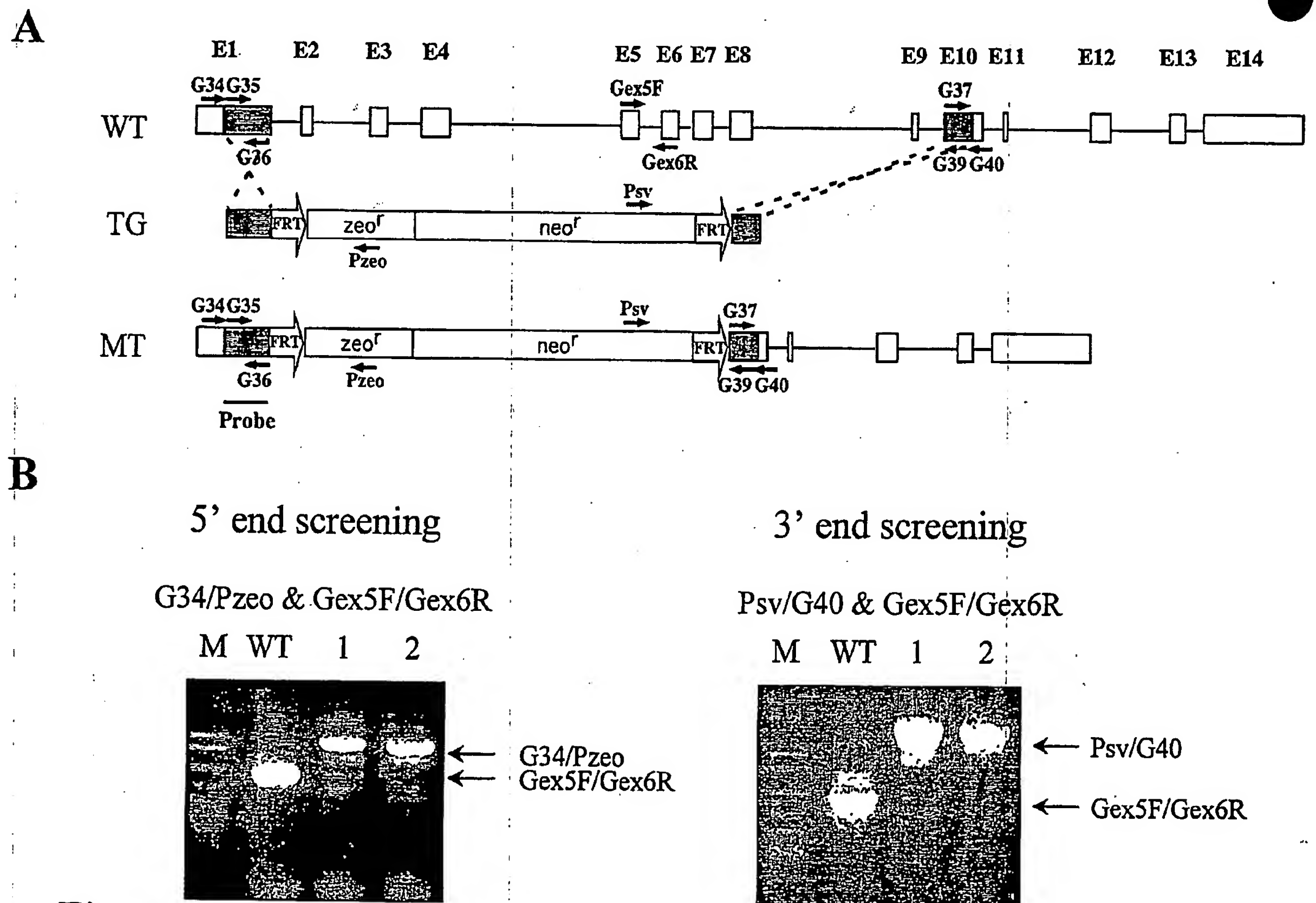
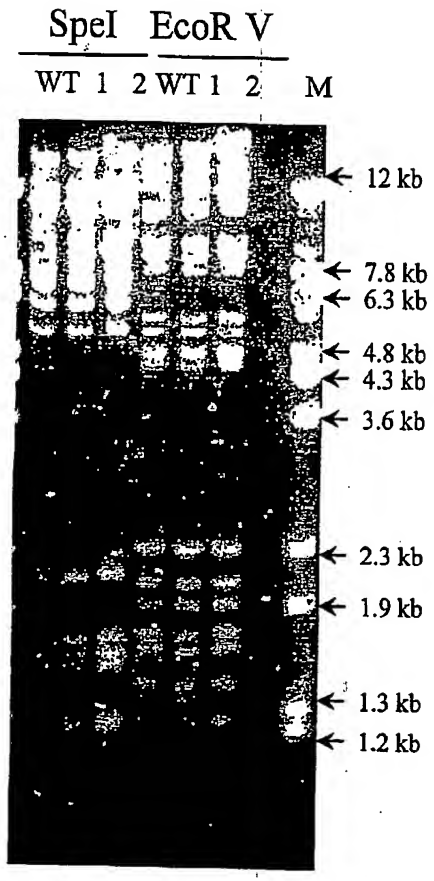
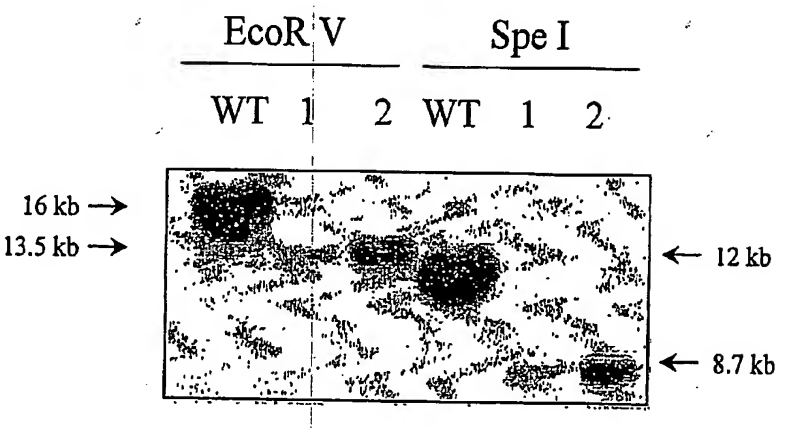

Wild Type BAC
Linear DNA
Modified BAC
Homologous recombination
Cm^r
Zeo^r
P1
P2
P3
P4
BAC vector
Genomic Insert
Exon to be disrupted
Homologous sequences
Nonhomologous sequences
FRT flanked zeo^r/neo^r cassette
PCR primers in the zeo^r/neo^r cassette
P3 & P4
PCR primers in the nonhomologous region
Restriction endonuclease recognition site

Figure 1E



Figures 2A and 2B





Figures 2C and 2D

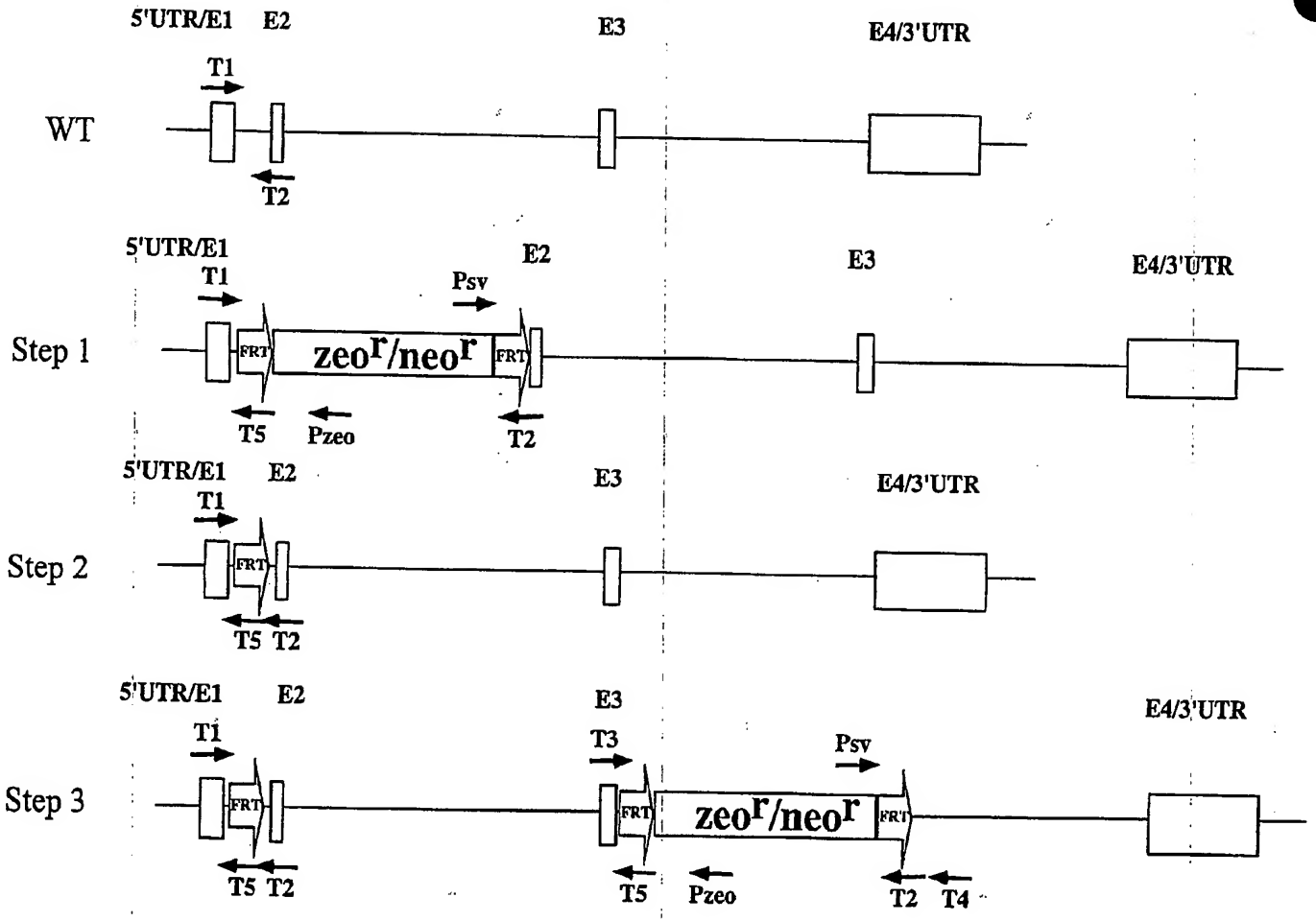

5'UTR/E1
E2
E3
E4/3'UTR
T1
WT
T2
5'UTR/E1
E2
E3
E4/3'UTR
T1
Psv
Step 1
FRT
zeo^r/neo^r
FRT
T5
Pzeo
T2
5'UTR/E1
E2
E3
E4/3'UTR
T1
Step 2
FRT
T5
T2
5'UTR/E1
E2
E3
E4/3'UTR
T1
T3
Psv
Step 3
FRT
FRT
zeo^r/neo^r
FRT
T5
T2
Pzeo
T2
T4

Figure 3A

B

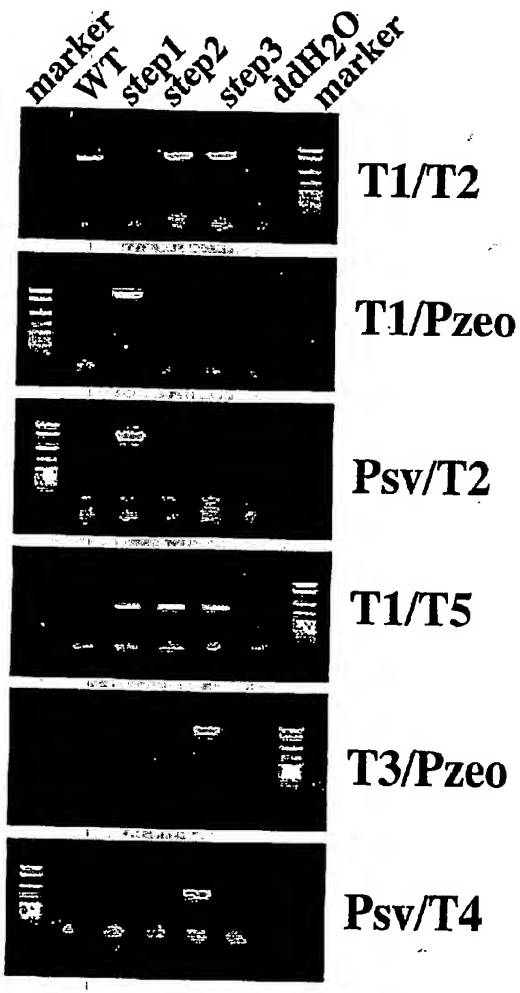
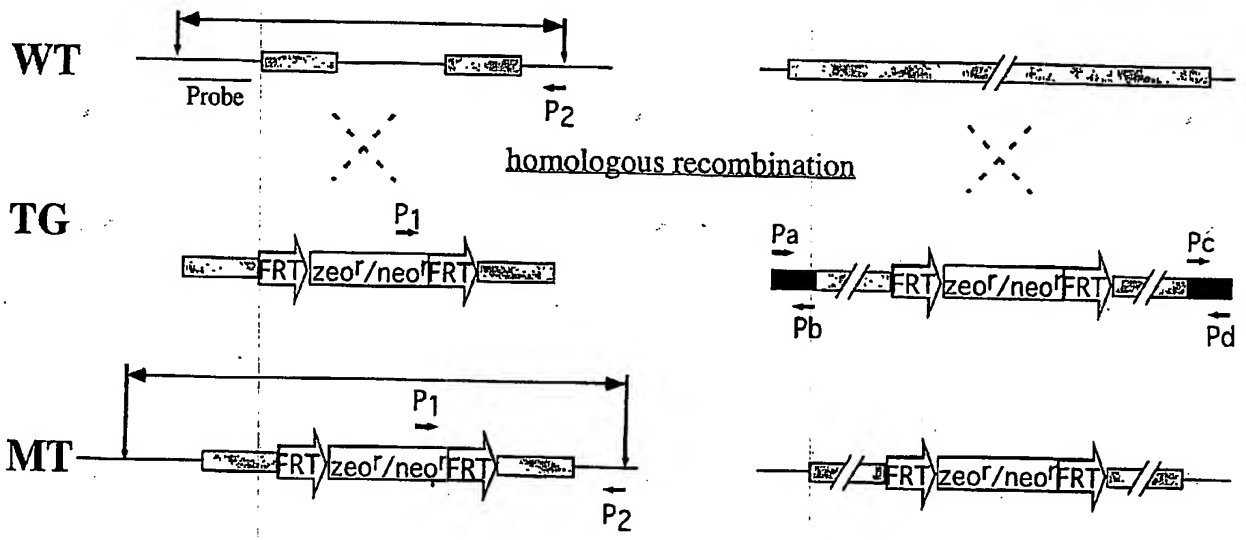


Figure 3B

Figures 4A and 4B



Assay	Results
Genomic PCR w/ P1/P2	——
Southern blotting	—— MT —— WT

Assay	Results
PCR w/ Pa/Pb or Pc/Pd	no product
FISH	

random insertion

Assay	Results
Genomic PCR w/ P1/P2	no product
Southern blotting	—— WT

Assay	Results
PCR w/ Pa/Pb or Pc/Pd	——
FISH	

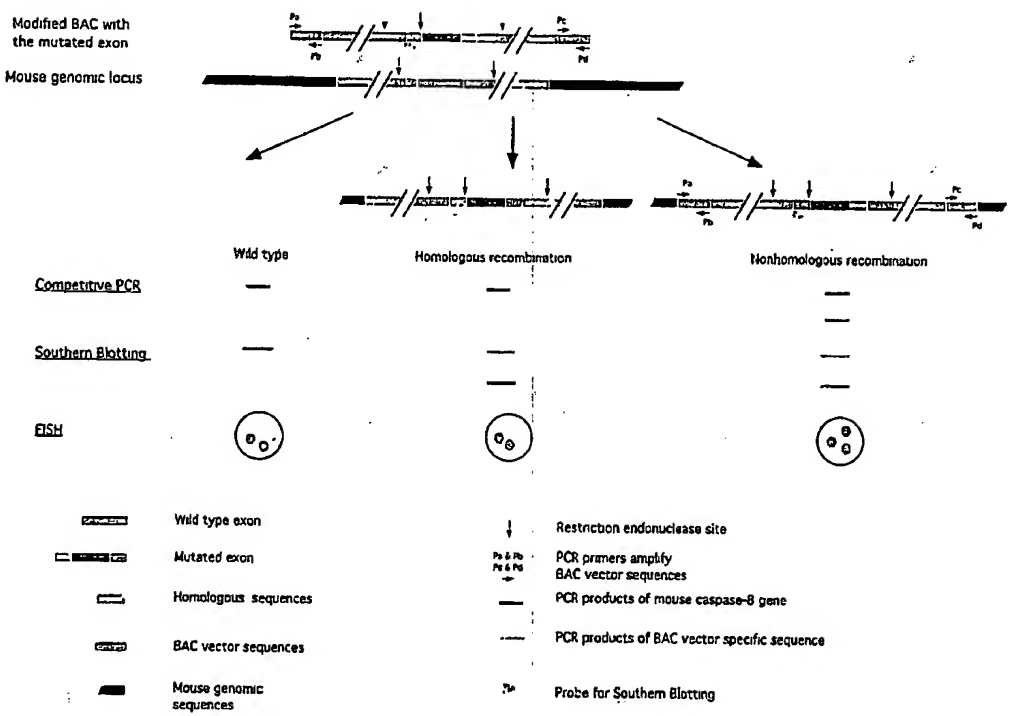
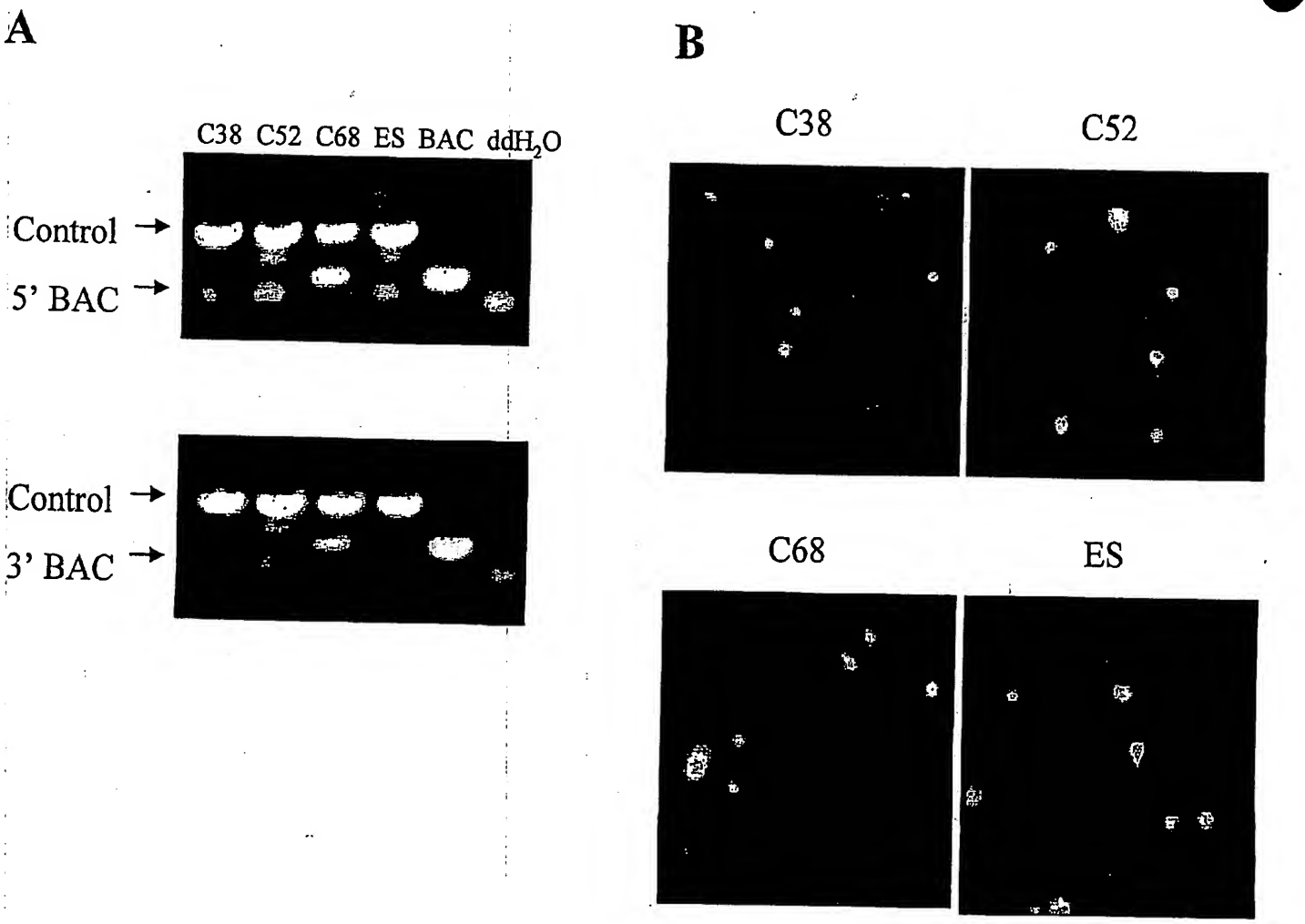


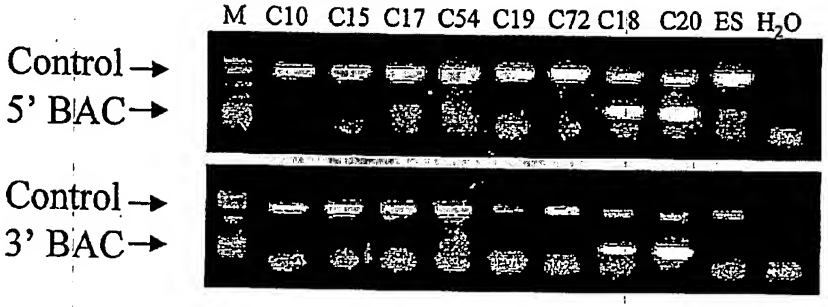
Figure 4C

Figures 5A and 5B

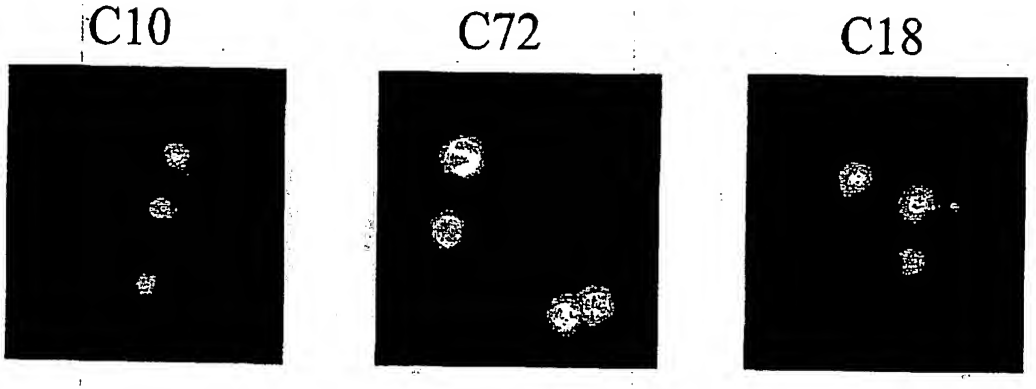


Figures 6A and 6B

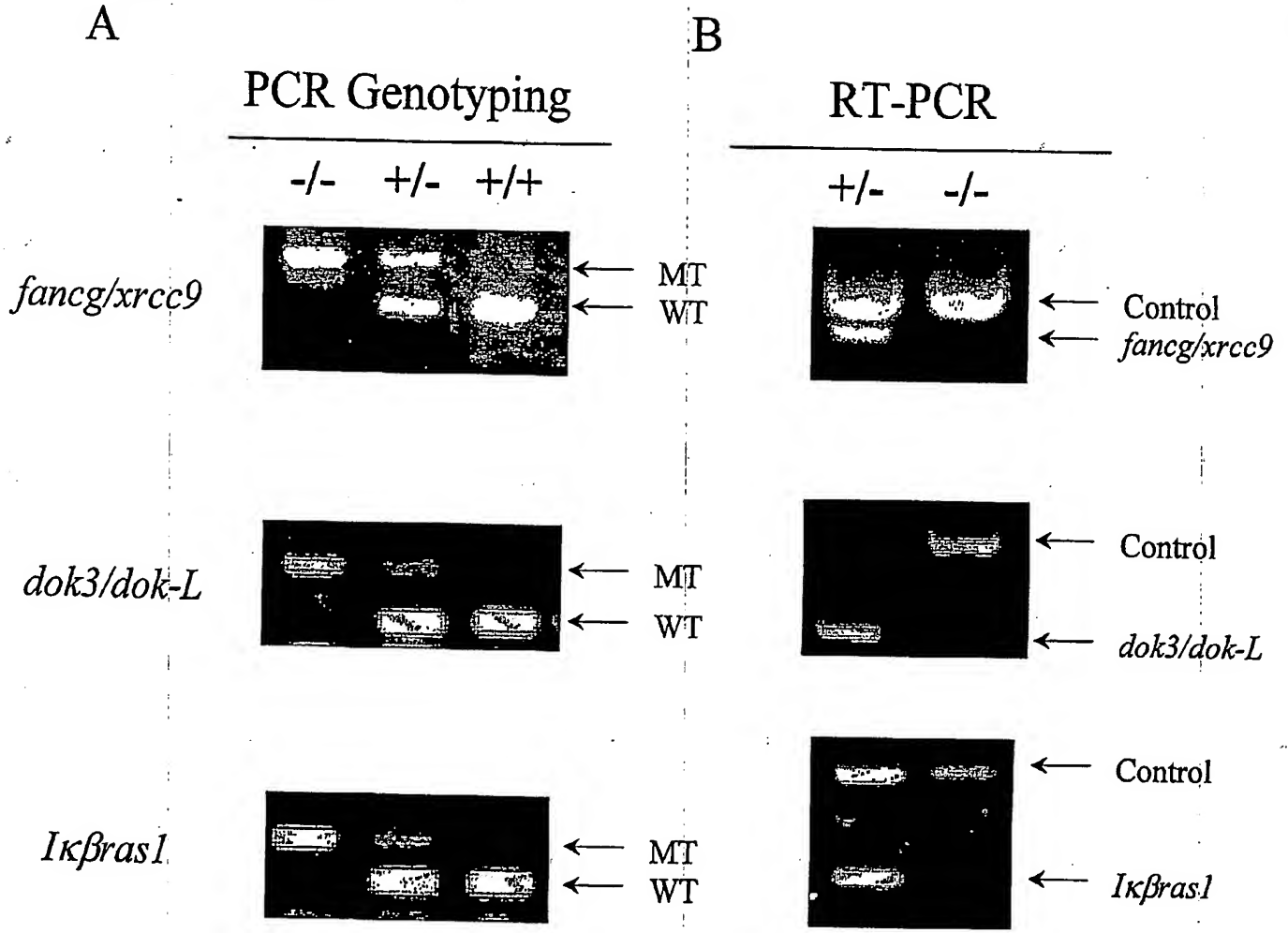
A



B

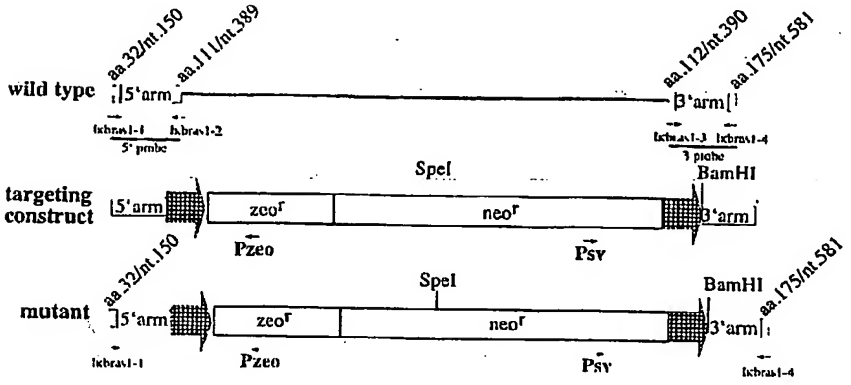


Figures 7A and 7B

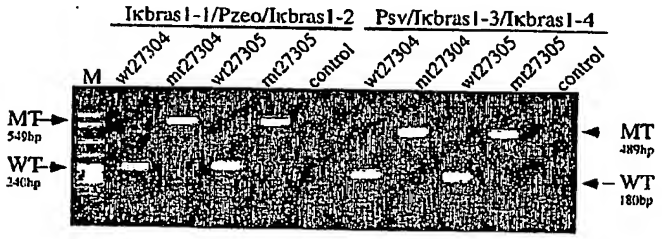


Figures 8A-8D

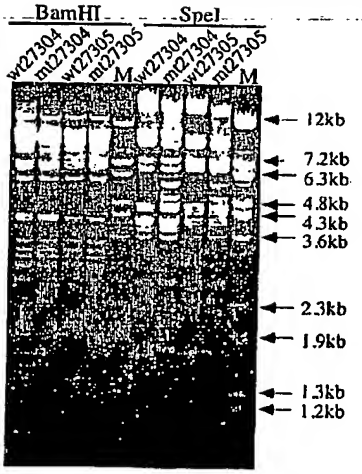
A



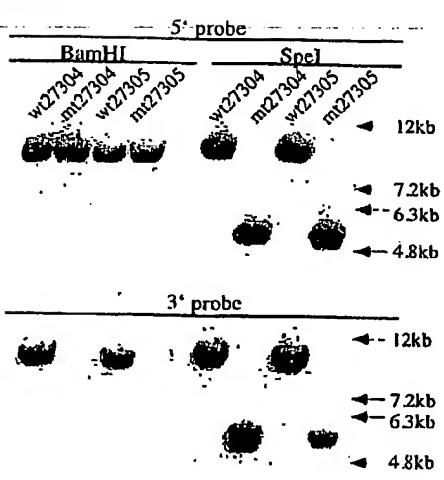
B



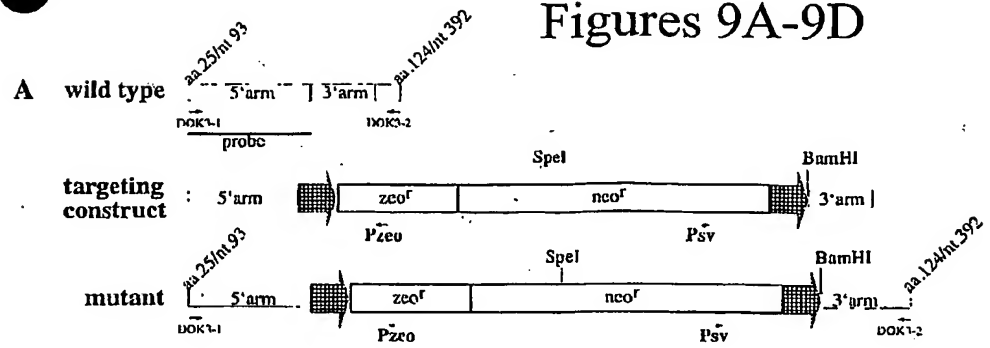
C

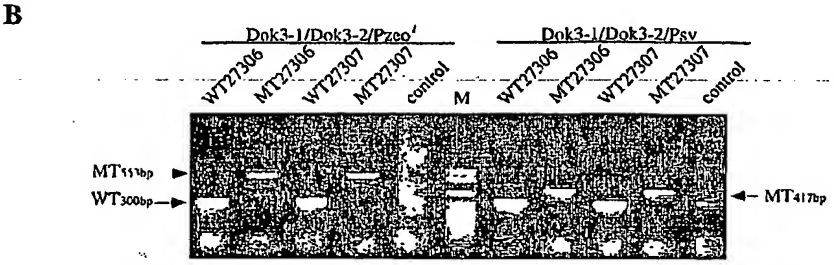


D



Figures 9A-9D





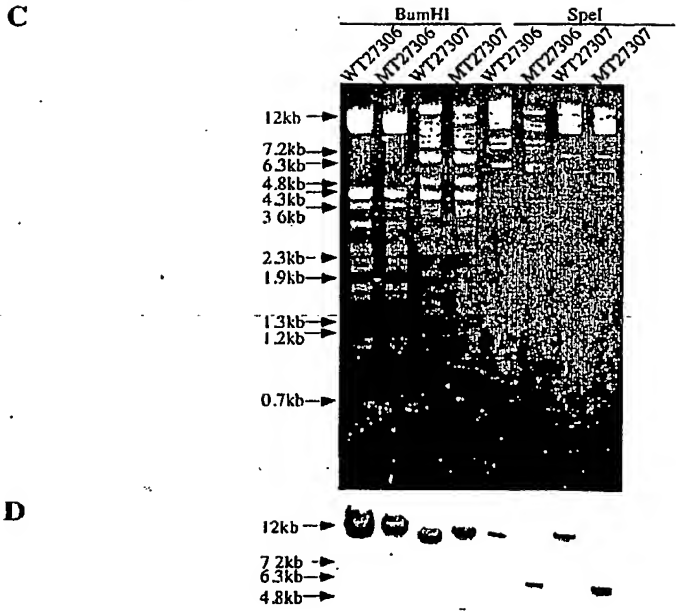


Figure 10

Gene/Clone	5'arm (nt)	3'arm (nt)	Deletion (nt)	Efficiency*
fancg/xrcc9	480	260	4900	100%
Iκβras1.#1	185	138	unknown	30%
Iκβras1.#2	185	138	unknown	25%
tab2.#1	213	340	869	30%
tab2.#2	213	340	869	50%
pag.#1	105	373	no	100%
pag.#2	105	373	no	58%
pag.#3	105	373	no	100%
dok3/dok-L.#1	188	93	no	42%
dok3/dok-L.#2	188	93	no	30%
bruce	50	50	no	100%

•The efficiency was calculated based on nested PCR screening of 24 ampicillin and chloramphenicol double resistant colonies at the 3' integration site.